Mate choice in giant panda (*Ailuropoda melanoleuca*)

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ABSTRACT. Giant pandas are difficult to breed in captivity due to low oestrus and mating rate, high cub mortality and diseases. Thus, improving the mating success of giant pandas in captivity is an important conservation issue. After observations on eleven female and three male giant pandas from Beijing Zoo, Lanzhou Zoo, Chengdu Zoo and Giant Panda Breeding Center during their mating season in 2000~2001, we found that mate choice plays an important role in mating success. Both male and female pandas actively chose their mates. Successful copulation only occurred in those males and females that both showed high frequencies of courting behaviour towards the opposite sex. Of those cases that only a male or female showed one-sided high or low frequency of courting behaviour in the keeper-arranged panda pairs in random order, no copulation was observed. Only three out of twenty-four paired pandas successfully copulated. All three impregnated female pandas in this study bore cubs. This indicated that mating choice was one of the important factors resulting in unsuccessful copulation and failure to reproduce. We recommend that attention be paid to the mate choice in giant panda when breeding pandas in pens are paired for reproduction in the future.

KEY WORDS: giant panda (*Ailuropoda melanoleuca*), mate choice, natural mating, courting frequency, survival fitness

INTRODUCTION

It is difficult to breed the giant panda in captivity due to their low reproduction rate, high cub mortality rate and diseases (Hu, 1988; Peng et al., 2001a; b). Up to September 1, 1997, 454 giant pandas had been maintained in captivity in the world. Among them were 152 males, 210 females, and 92 of unknown sex (cubs that died before their sex was identified) (Xie & Gipps, 1997). However, only 34 (12 males, 22 females) of those pandas ever produced offspring by natural mating or artificial insemination (Xie & Gipps, 1997; Huang et al., 1999). Currently, there are only six adult male giant pandas kept in captivity, five wild born and one born in captivity that can naturally copulate with adult female pandas (Lindburg et al., 1997; Huang et al., 1999). Moreover, to date only one adult male and six females born in captivity have successfully produced offspring (Xie & Gipps, 1997). How to improve the mating success of giant pandas in captivity, especially of male pandas, is an important conservation issue. We therefore needed to study the reproductive biology, and especially the reproductive behaviour of pandas, because the main reason for failed reproduction in pens is the Jack of successful courting and copulation. So, we studied the courting behaviour and mating success of the arranged pairing in giant pandas from Beijing Zoo, Lanzhou Zoo, Chengdu Zoo and Chengdu Giant Panda Breeding Center during their mating season in 2000~2001.

MATERIALS AND METHODS

We observed the courting frequencies of eleven adult females and three adult males. All observed giant pandas were healthy and 10 of 14 had mated naturally in a previous breeding season (Table 1). In order to increase the genetic variability of giant pandas in captivity and avoid inbreeding, two adult female pandas (Stud# 403 and 421) from Beijing Zoo and one female panda (Stud# 407) from Lanzhou Zoo were transported to Chengdu Zoo, and two adult male pandas (Stud# 369 and 345) from Beijing Zoo were transported to Chengdu at the end of February in 2000 and 2001. Male and female panda were paired by the keepers according to studbook, origin and birth location to avoid inbreeding only when both were observed to present courting or rutting behaviour during their mating season. Twenty-four groups were paired in the study, identified as A, B, …, W, X (Table 2). We scanned each paired group as soon as they were paired every morning from 8:00 am to 11:00 am at 10-minute intervals from March 1st to April 30th in their brief breeding season in 2000-2001 and recorded the courting frequency of a male towards a female or a female towards a male.

TABLE 1

Reproductive records of the giant panda in the study.

Name	Stud #	Sex	Birth date (MM/DD/YY)	Origin	Location	Reproduction history before the study
Le Le	320	Female	9/8/1986	Captive born	Beijing Zoo	Gave birth to 7 cubs (3 twins) by natural mating and artificial insemination.
Ying Ying	369	Male	8/15/1991	Captive born	Beijing Zoo	Had a record of natural copulation last year.
You You	345	Male	6/23/1988	Captive born	Beijing Zoo	Has oestrus every year after sexual maturation, but never successfully copulated with a female. His semen is artificially collected every year.
Ji Ni	403	Female	11/4/1993	Captive born	Beijing Zoo	Had her first oestrus last year, naturally mated but not pregnant. Had her second oestrus this year.
Niu Niu	421	Female	9/5/1995	Captive born	Beijing Zoo	Had her first oestrus last year, naturally mated but not pregnant. Had her second oestrus this year.
Qing Qing	278	Female	9/9/1984	Captive born	Chengdu Zoo	Gave birth to 8 cubs (2 twins) by natural mating or artificial insemination.
Ha Lan	287	Male	8/1984	Wild born	Chengdu Zoo	Fathered 7 cubs (3 twins) by natural mating or artificial insemination with his semen.
Cheng Cheng	297	Female	9/24/1985	Captive born	Chengdu Breeding Center	Gave birth to 5 cubs (1 twins) by natural mating or artificial insemination.
Bing Bing	314	Female	8/6/1986	Captive born	Chengdu-Breeding Center	Gave birth to 8 cubs (2 twins) by natural mating or artificial insemination.
Li Li	387	Female	9/3/1992	Captive born	Chengdu Breeding Center	Did not have oestrus due to poor health. However, Li Li had her first oestrus this year.
Er Yatou	401	Female	9/19/1993	Captive born	Chengdu Zoo	Gave birth to 1 set of twins by natural mating and artificial insemination last year.
Mei Mei	408	Female	8/31/1994	Captive born	Chengdu Breeding Center	Gave birth to 1 set of twins by natural mating and artificial insemination last year.
Jiao Zi	425	Female	8/21/1995	Captive born	Chengdu Breeding Center	Had her first oestrus this year.
Shu Lan	407	Female	8/31/1994	Captive born	Lanzhou Zoo	Had her first oestrus this year.

Courting behaviour in the study was defined as one or all of the following behaviours: (1) a panda approached a sexual partner forwardly, and presented estrous or rutting behaviours, such as shaking head, urinating/defecating, rubbing anogenital area etc.; (2) he or she bleated "Mie, Mie", stared at the partner, sniffed the urine, faeces and the scent mark left by the partner; (3) a panda tried to scratch the partner in order to attract his or her attention. A female was paired with a male if she firstly showed no signs of aggression towards the male partner, then she perhaps raised her hindquarters, erected her tail and showed the anogenital region to the male as he courted her, and she finally accepted his mounting. While the male mounted the female inserted his penis and thrust, both pandas bleated during the copulation, and the female vocalized quavery moans as the male ejaculated. If the male's penis entered the female panda's vagina and we later heard the high chirp cries of the female, we recorded the mating as a successful copulation.

If the pandas were paired and they started to bite and attack each other, or if the male or female showed little courting towards the other, or if they seldom approached each other in the pen and never copulated, the keeper finally separated the two pandas. Then, we recorded the pairing as a failed copulation.

We recorded the duration of the copulation, as well as mating success or failure during their copulation. Subsequently, we monitored whether the female pandas became pregnant and gave birth. We used the Mann-Whitney U test to check the difference between the courting behaviours of the male and female panda for each paired group separately. Kruskal-Wallis H tests were used to check the difference in mating and courting frequencies among the groups over all paired experiments.

TABLE 2

Observations (every 10-minute period during their pairing) on courting frequency and mating success in pandas paired by their keepers in random order according to Studbook and birth location to avoid inbreeding.

Group	Male Stud #	Female Stud #	Courting/10 min.		Date of mating (mm/dd/yy)	Copulation duration (min)	Parturition	Kruskal-Wallis H Test
			Male	Female				
A	287	314	0.48 ± 0.08^{h}	0.48 ± 0.08^{h}	3/11~13/00	2±1(2)	2 cubs	*
B	287	278	0.58 ± 0.10^{h}	0.56 ± 0.08^{h}	3/25~28/00	8±2(3)	1 cub	*
C	287	403	0.49 ± 0.09^{h}	0.17 ± 0.05^{l}	4/12~14/00	0	0	
D	287	421	0.48 ± 0.09^{h}	0.07 ± 0.05^{l}	4/10~12/00	0	0	
E	369	297	0.44 ± 0.07^{h}	0.09 ± 0.03^{l}	3/30~4/2/00	0	0	
F	369	407	0.18 ± 0.05^{l}	0.59 ± 0.09^{h}	4/06~08/00	0	0	
G	369	401	0.17 ± 0.04^{l}	0.17 ± 0.04^{l}	4/19~21/00	0	0	
H	345	408	0.14 ± 0.05^{l}	0.16 ± 0.04^{l}	4/12~14/00	0	0	
I	345	425	0.06 ± 0.03^{l}	0.04 ± 0.04^{l}	4/16~18/00	0	0	
J	345	387	0.04 ± 0.03^{l}	0.06 ± 0.03^{l}	4/06~08/00	0	0	
K	369	320	0.64 ± 0.10^{h}	0.58 ± 0.10^{h}	3/27~29/00	4±1(3)	2 cubs	*
L	345	320	0.19 ± 0.04^{l}	0.16 ± 0.05^{l}	4/01~03/00	0	0	
M	369	297	0.55 ± 0.11^{h}	0.12 ± 0.03^{l}	3/21~23/01	0	0	
N	345	297	0.48 ± 0.11^{h}	0.19 ± 0.06^{l}	3/25~28/01	0	0	
O	369	401	0.19 ± 0.04^{l}	0.21 ± 0.05^{l}	4/11~13/01	0	0	
P	345	408	0.21 ± 0.09^{l}	0.19 ± 0.06^{l}	4/16~18/01	0	0	
Q	369	408	0.12 ± 0.03^{l}	0.08 ± 0.04^{l}	4/20~22/01	0	0	
R	345	387	0.14 ± 0.04^{l}	0.18 ± 0.06^{l}	3/16~18/01	0	0	
S	287	403	0.36 ± 0.08^{h}	0.11 ± 0.04^{l}	3/29~4/2/01	0	0	
T	287	421	0.35 ± 0.09^{h}	0.09 ± 0.04^{l}	4/08~10/01	0	0	
U	287	407	0.27 ± 0.08^{l}	0.19 ± 0.06^{l}	4/17~20/01	0	0	
V	369	407	0.19 ± 0.07^{l}	0.52 ± 0.14^{h}	4/23~25/01	0	0	
W	369	425	0.06 ± 0.03^{l}	0.04 ± 0.04^{l}	4/26~28/01	0	0	
X	345	425	0.16 ± 0.03^{l}	0.25 ± 0.03^{l}	4/22~24/01	0	0	
						Copulation rate: 12.5%	Parturition rate: 100%	

Notes:

(1) Superscript *"h"* denotes *high* frequencies of courting, and superscript *"l"* denotes *low* frequencies of courting.

(2) *h-l* denotes significant differences in courting frequencies between male and female in the same paired group ($P<0.05$; Mann-Whitney U Test).

(3) *h-h* or *l-l* denotes no statistical differences between male and female in the same paired group ($P>0.05$; Mann-Whitney U Test).

(4) Number of matings is shown in the parentheses. "0" indicated the pandas failed to copulate and couldn't become pregnant.

(5) *" * "* denotes significant difference in mating and courting frequencies among the groups over all paired experiments. ($P<0.05$; Kruskal-Wallis H Test).

RESULTS

The courting frequencies of every male or female in each paired group were scanned 216 times. We found a significant difference of courting frequency between males towards females or females towards males ($P<0.05$; Table 2). This indicated that attitudes of males or females to the planned mate could be described as like or dislike. Further, we found that only when both male and female in a paired group showed high frequencies of courting behaviours towards each other did they successfully copulate. When the male and female in a paired group both showed low frequencies of courting behaviours or showed one-sided high or low frequencies of courting behaviours, they failed to copulate.

We noted three types of outcome: First, the male and female panda showed different frequencies of courting behaviour: either the male actively approached and courted the female, but the female ignored him and refused to copulate, or the female actively approached and courted the male but the male declined to mount the female. Second, both male and female showed equal but low frequencies of courting behaviour, they evaded each other and did not copulate. Third, both male and female showed high frequencies of courting behaviour and successfully copulated. Overall the rate of successful copulation was 12.5%. Because the females of all three pairs that successfully copulated became pregnant and bore cubs, the parturition rate was 100%.

Courting frequencies of both males and females in Groups A, B and K were significantly higher than those of other groups (Fig. 1). This indicated that both males and females in Groups A, B and K were interested in each other, and only they mated favourably and successfully. The males and females in other groups were either uninterested in each other, or exhibited one-sided interest only or failed to copulate.

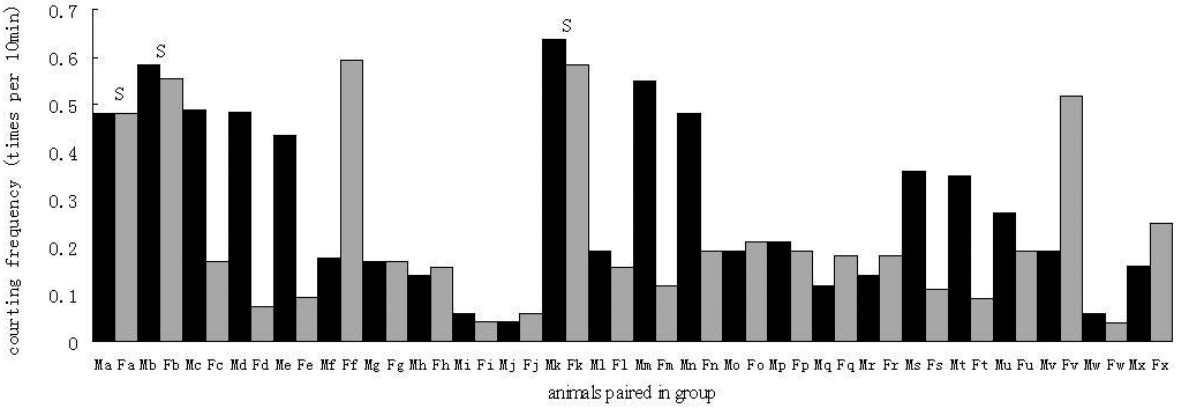

Fig. 1. – Frequencies of active courting of male or female giant pandas during their mating season (times/10min).

Notes:
(1) *"Ma"* (left dark column), *"Fa"* (right grey column) denotes frequencies of active courting of male or female respectively towards the opposite sex in Group A. Likewise, Mb, Fb for Group B, etc.
(2) *"S"* denotes male and female successful copulation and parturition.

DISCUSSION

How to improve the mating and breeding success of giant panda in captivity is a key issue in maintaining the genetic diversity and propagating captive panda populations. Aiming to achieve a high breeding rate in captive panda populations, the rational regime is that the panda keepers in zoos arrange the pairing of the male and female pandas in pens during the breeding season. However, the practice is never assessed even though the pandas in pens have a low mating success.

During the breeding season, giant pandas communicate information about their status of estrous and emotion to each other by olfactory, auditory, visual and touch signs (SCHALLER, 1993; HU, 1988). For example, they transfer the information of their estrous and emotional status to the opposite sex by defecating, urinating, scent-marking, bleating, courting and staring. If they are interested in the the opposite sex, then they will frequently court and show intimate behaviours, which can induce successful copulation. If they are not interested in the partner, they will show less courting behaviour and ignore them. When pandas in zoos were penned together, male or female pandas frequently approached, sniffed and courted their partner. Such courting behaviours pass breeding information and stimulate mating drive in their partners (HU, 1988; MEFFE & CARROLL, 1994; GAO & PU, 1994). When individuals of both sexes show a high frequency of courting behaviour, such a process may ultimately lead to mating.

Giant pandas primarily live solitarily and territorially during the non-breeding season in the field. Males and females seldom contact each other (HU et al., 1985; Hu, 1990a; 1990b). Only during breeding season do the giant pandas start to search for mates frequently. Males may gather and compete for mates. On one occasion, five males chased and mated with a female in turn in the Wolong natural reserve, thus, WANG (1987) thought that female pandas play a passive role in copulation. GAO & PU (1994) also thought the male giant pandas do not choose mates during the breeding season. They thought that mating success depends mainly on the number of healthy, sexually matured males in a population. However, in this study, we found out that male and female giant pandas have diverse behaviour. If only the male panda showed high courting behaviour to a female panda but the female panda had no or little response to the male's courting, then they would not copulate, or vice versa. Giant pandas appear to be selective towards their sexual partners, either male or female pandas refusing to mate with a sexually mature partner when he or she is not interested in courting. Only those, who show high corresponding courting behaviour towards each other will ultimately mate. Coordination between mates is essential for a successful mating (KLEIMAN, 1983). In this study, males or females all chose healthy partners with a mating history, which implies that inter-sexual selection is an important factor in the reproduction of giant panda. Moreover, HU (1990b) observed that one estrous female was actively courting one male but ignored the other males. He thought that not only mate competition but also mate choice occurred in giant pandas.

Active mate choice has been inferred from the observation that individuals visit several prospective mates but choose only one (or a subset) of them and reject the remaining ones (GIBSON & LANGEN, 1996). Most studies report that animals, over a wide taxonomic range, engage in a process of active choice while searching for mates (PARKER, 1983), including insects (MOORE, 1989), crustacea (BACKWELL & PASSMORE, 1996), fish (WARNER, 1995), frogs (RYAN, 1985), birds (GIBSON, 1996; FISKE &

KÅLÅS, 1995; RINTAMÄKI et al., 1995; DALE et al., 1990; PETRIE et al., 1991; BENSCH & HASSELQUIST, 1992; TRAIL & ADAMS, 1989) and mammals (BYERS et al., 1994). In our study, we also found that giant pandas actively choose their mates during their breeding season. Not only the females actively chose the males, but also the males actively chose the females. Finally, only those males and females who showed high frequencies of courting behaviour to each other mated.

Sexual selection has been a major focus of research in evolutionary biology since Darwin noted that mate choice confers an immediate advantage to preferred individuals. The origin of mate preferences remains controversial, and the operation of mate choice as a force independent of natural selection is still disputed (REAL, 1990). Whatever its origin, when certain potential mates are differentially attractive, those characteristics conferring the advantage increase in frequency within the population (REAL, 1990; 1991). Sexual selection depends on differential patterns of mate preference and choice. PARKER (1983) distinguishes three types of mate choice: (1) both sexes are non-discriminating in their choice of mates; (2) one sex is passive and non-discriminating, but the other sex engages in active choice; and (3) both sexes are discriminating and engage in active choice. The first two types are treated by traditional optimal-diet theory, and the third is modelled as an evolutionarily stable strategy (REAL, 1990; 1991). Giant panda may belong to the third type.

When both sexes choose their mates, low quality (less desirable) individuals should be less discriminating (GIBSON & LANGEN, 1996). Pandas in captivity have a limited number of mates to choose from and most pandas declined to copulate with mates they disliked. In our study, only three out of twenty-four paired pandas successfully copulated, the success rate being 12.5%. This indicates that mate choice was one of the important factors in unsuccessful copulation and failure of reproduction. We should pay attention to mate choice in giant panda when we pair breeding pandas in pens in the future. Moreover, only three mated female pandas in this study bore cubs, and all others failed. Generally, female pandas reproduce every 1.5~2.5 years after sexual maturity in the field, meaning that they only produce 6~8 litters throughout their lifetime (HU, 1988; 1990a).

Research and conservation biology have become much more important to zoos ever since it became clear that the zoo is a place for the long-term management of many endangered species rather than just a place for the short-term keeping of exotic exhibits (HARDY & KRACKOW, 1995). Most of the research carried out in zoos has therefore an "applied" focus towards these goals (ZHANG et al., 1996; DING et al., 1998; MASUI et al., 1989; ZHANG et al., 1994; MAINKA & ZHANG, 1994; CHEN et al., 1994; DIERENFELD et al., 1995). We should compare the behaviour of wild and captive pandas to reveal any differences in reproductive behaviour and try to enhance copulation rate in captivity. Unfortunately, this has rarely been done because of the difficulty of work in the wild. Zoos could thus be a useful yet under-used resource for behavioural research. However, only a small number of animals have been bred in captivity, thus small group size leads to small sample size for research. In our study, we only studied 24 groups of paired pandas because of limited availability of animals, and even the number of males was far less than that of females. So, it is necessary to make more male pandas become founders, or else, the shortage of reproductive males will increase the inbreeding probability and decrease the genetic diversity of the captive population.

Captive pandas have been separated into tens of very small populations in the world, some zoos even having only one or two pandas. Furthermore, all captive pandas seldom have the chance to meet and copulate, and around eighty percent of pandas in captivity never reproduced before they died (PENG et al., 2001a; b). So, we advise that all reproductive pandas in captivity should be gathered together to let them have their chances to choose their mates during the breeding season. This is required to ensure successful breeding for the limited number of pandas in captivity.

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Diversity and dynamics in a community of small mammals in coastal Guinea, West Africa

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ABSTRACT. In order to investigate dynamics and reproduction in *Mastomys erythroleucus* inhabiting a high rainfall area in coastal Guinea, West Africa, a small mammal study was carried out through a 1-year longitudinal survey. Sampling was by standardized trapping in houses, cultivations, forest and savanna. Identification of the small mammals was based on morphology, and by molecular technique for sibling species. As a part of a larger survey on reservoirs of Lassa virus in Guinea, 106/289 specimens were screened for arenavirus and were found negative. The most abundant species was *M. erythroleucus* (46%) which occurred in all habitats, with a preference for savanna close to the cultivations. Its reproduction was seasonal and lasted for 8 months, beginning in the early rainy season and finishing in the early dry season. It was syntopic with *Lophuromys sikapusi* (13%) and *Praomys rostratus* (10%), which probably migrated from forest/orchards to cultivations in the late rainy season. Reproduction was high in many species in the late rainy season, but *P. rostratus* seemed to reproduce actively in the dry season, in contrast to *L. sikapusi*. Pygmy mice, *Mus (Nannomys*) spp., were abundant in the early rainy season only. The high species richness (14) is explained by the combined influence of Sudanian-Guinean-Congolian habitats. The role of the absence of bush fires is also debated in that context.

KEY WORDS : abundance, diversity, Lassa virus, *Lophuromys sikapusi*, *Mastomys erythroleucus*, *Mus* (*Nannomys*) spp., *Praomys rostratus*, reproduction, season.

INTRODUCTION

We carried out a terrestrial small mammal survey to investigate the reservoirs of Lassa virus in Guinea, West Africa. The human disease, Lassa Fever (LF) is definitely linked with the presence of *Mastomys natalensis* (Smith, 1834), which is absent in coastal Guinea (LECOMPTE et al., 2006). In that region, only *Mastomys erythroleucus* (Temminck, 1853) was present and it has never been found to be infected by Lassa virus, justifying the low endemic zone described by LUKASHEVICH et al., (1993). In Africa, *M. erythroleucus* is distributed from Senegal to Ethiopia, and from south Sahara to the Equator, partially overlapping with the distribution of *M. natalensis* (GRANJON et al., 1997). In Senegal particularly, numerous studies have been carried out during the last 30 years, describing many aspects of population ecology: abundance, reproduction, growth, diet, home range, behaviour and parasite infections (HUBERT et al., 1977; HUBERT, 1982; HUBERT & DEMARNE, 1981; HUBERT & ADAM, 1985; DUPLANTIER & GRANJON, 1988; DUPLANTIER et al., 1996; SÈNE et al., 1996; BROUAT et al., 2007). Our exploration was an opportunity to study the population ecology of *M. erythroleucus* in a more humid area than that of the earlier studies in Senegal. This area was chosen because of a similar habitat, here a Guinean forest-savanna mosaic (WHITE, 1983), to those investigated in a high endemic zone (FICHET-CALVET et al., 2007), which have a similar annual rainfall of around 1600-1900mm per year. In these climatic conditions, do the dynamics and demography vary seasonally as they do in drier zones where the rainy season is shorter? A study in 1936-37 in Freetown, Sierra Leone, showed an extensive reproductive period throughout the year in *M. erythroleucus* but many specimens trapped in the "native huts" and outside were included in the analysis, possibly showing a longer period of sexual activity due to increased food availability (BRAMBELL & DAVIS, 1941).

Because faunal assemblages could differ in relation to season, we present the diversity of this small mammal community, based on three sampling periods: two in the rainy season and one in the dry season. Through this one-year longitudinal survey, the spatio-temporal dynamics of the most abundant species accompanying *M. erythroleucus* were also investigated.

MATERIALS AND METHODS

Study site

The village of Gania (10°03'58"N, 12°32'27"W, 240m asl), prefecture of Kindia, was investigated in June 2004 (early rainy season), October 2004 (late rainy season) and February 2005 (dry season). The annual mean rainfall at Kindia, the closest station, was 1953mm between 1961 and 1990 (www.meteo-guinee-conakry.net, rainfall data

not available during this study). The rainy season lasts from April to November.

Of the 3465 trapping nights during this period, 900 were in houses, 1545 in cultivations, 780 in forest and 240 in savanna. The cultivated land comprised crops of millet, maize, peanuts, ochras, cucumbers, sweet potatoes, cassava and rice fields, 1-year fallow lands, and pastures. Forest comprised orchards (bananas, mango, orange, guava) mixed with native trees (*Afzelia africana*, *Anosophyllea laurina*, *Ceïba pentandra*, *Cola acuminata*, *Combretum micranthum*, *C. paniculatum*, *Lophira lanceolata*, *Parkia biglobosa*, *Pterocarpus erynaceus*, *Sterculia tragacantha*). Savanna was a more open habitat, such as wooded fallow land and grassland. In each of these habitats, the traps (Sherman LFA live trap, H.B. Sherman Traps, Inc.) were set for three consecutive nights with 100 traps in houses, 160-180 traps in cultivations and 80 traps in forest and 20-40 in savanna. The sampled houses were distributed along a line through the village, with two traps set in each room. Usually, the houses are rectangular with a mud-brick wall delimiting several rooms, and covered with a roof of corrugated galvanized iron. A maximum of 12 traps per house were set. In cultivations, forest and savanna, the traps were set in lines of 100m with 20 traps placed singly, each 5m apart. Traps were baited with a mixture of peanuts, dry fish and wheat flour.

Autopsies, identification of the species and demographic parameters

Because this study was part of a larger survey on the reservoirs of LF in Guinea, trapped rodents and shrews were handled using P3 standard rules (Mills et al., 1995) and killed by lethal dose of anesthetic (fluorane). To avoid any contamination of the field workers and the material, the autopsies were performed *in situ*, at a cleaned place under the trees and near the village. The rodents were described morphologically, weighed, measured (length of head and body, tail, hindfoot and ear) to enable a preliminary identification according to the keys published for West Africa (Rosevear, 1969; Duplantier & Granjon, 1993). Individuals of *Lophuromys* were morphologically assigned to be *L. sikapusi* (Temminck, 1853) and the identity of six specimens was confirmed by cytochrome b (1140pb) sequence. According to the recent systematic revision of the genus *Praomys* (Lecompte et al., 2002a; Nicolas et al., 2005; Nicolas et al., 2008), two species exist in Guinea, the smaller form *P. tullbergi* (Thomas, 1894), and the larger form *P. rostratus* (Miller, 1900). Because the adult *Praomys sp.* in our study had measurements (weight: 49.0±12.0g, head and body: 127.5±10.1mm, tail: 152.9±13.0mm, hindfoot without claws: 25.0±1.0mm, n=44) consistent with those described for *P. rostratus* in Côte d'Ivoire (Van der Straeten & Verheyen, 1981), and also because molecular determination was confirmed by sequencing cytochrome b (1140pb) for four specimens, we consider all the specimens as belonging to that species in this study. *Mus* (*Nannomys*) is also a complex of morphologically very similar species living sometimes in sympatry, which thus has led to many mis-identifications. Hence, all trapped specimens were identified molecularly based on 490 pb of cytochrome b gene (Veyrunes et al., 2005). Species identification of shrews was based on morphological and cranio-dental characters.

The weight of the desiccated eye lens ELW gives the best indication of age for small mammals (Lord, 1959; rev in Morris, 1971; Hubert & Adam, 1975). Eyes were removed and after preservation for a minimum of two weeks in 10% formalin, the lenses were extracted, dried for two hours at 100ºC, and weighed to the nearest 0.1mg. Females were examined for the diameter of the uterus, the number of foetuses or uterine scars, and for indications of lactation. They were classified as sexually active if they were pregnant or lactating or had recent scars in a large uterus. Males were classified as sexually active if the seminal vesicles were swollen and more than 30mm^2 in *Mus* (*Nannomys*), 100mm^2 in *Mastomys*, *Lophuromys*, *Lemniscomys* and *Uranomys*, 110mm^2 in *Rattus*, 90mm^2 in *Praomys* (formerly *Myomys*) *daltoni*, 120mm^2 in *Praomys rostratus*, and 280mm^2 in *Gerbilliscus* (formerly *Tatera*) *guineae*, in surface (length x width).

Data analysis

The species richness S represents the number of species in the community, and is weighted by the Shannon index of diversity (H), calculated as $H=-\sum p_i \ln p_i$, where p_i=number of individuals for each species/total number of individuals Shannon & Wiener, 1963. In theory, H increases with S, but practically does not exceed 5.0 in biological communities (Krebs, 1998). Evenness index (E) indicates how the species are distributed in the community, and is derived from H (E=H/lnS). The values range from 0 (one dominant species) to 1 (all species equally represented in the community). Here, S, H and E were calculated by habitat and season.

The abundance of the rodents was measured by abundance index (AI) calculated according to published methods as AI=∑(number of trapped rodents/length in m) x 100 (Spitz et al., 1974; Krebs, 1998), and for each species by habitat. As control, AI was compared to a mean trapping success (TS=∑(number of trapped rodents/∑trapping nights) x 100) for three consecutive nights. The habitats analysed statistically were: houses, cultivations, (nearby and remote), forest/orchards (nearby and remote) and savanna (wooded fallow land and grassland). Remote cultivations were separated from the houses by a corridor of proximal forest requiring at least 15min of walking. Remote forest was close to remote cultivations.

The variations of abundance index (AI) and age structure (ELW) were analysed using ANOVA with AI or ELW as the dependent variable and season (early rainy, late rainy, dry), and habitat (cultivations, forest/orchards, savanna) as independent variables. A post-hoc test (Scheffe's) was made to identify which pairs of means are significantly different. This analysis was performed using the SuperAnova software (Abacus Concepts, 1989). The variation of sexual activity was analysed through a multiple regression, including the independent variables as for the ANOVA's.

The comparison of proportions, describing the occurrence of the species by habitat, was made using a Chi2 test. Logistic regression and Chi2 were performed

through the software Statview 5 (SAS, Institute Inc. 1998).

RESULTS

Specific spatial distribution

We collected 289 small mammals of 14 species. These were: *Mastomys erythroleucus* (n=134), *Lophuromys sikapusi* (n=37), *Praomys rostratus* (n=28), *Rattus rattus* (Linnaeus, 1758) (n=28), *Gerbilliscus guineae* (Thomas, 1910) (n=15), *Uranomys ruddi* (Dollman, 1909) (n=10), *Mus* (*Nannomys*) *mattheyi* (Petter, 1969) (n=10), *M.* (*Nannomys*) *minutoides* (Smith, 1834) (n=6), *Lemniscomys striatus* (Linnaeus, 1758) (n=5), *Crocidura olivieri* (Lesson, 1827) (n=5), *C. buettikoferi* (Jentink, 1888) (n=4), *P. daltoni* (Thomas, 1892) (n=4), *Hylomyscus simus* (Allen & Coolidge, 1930) (n=2), and *L. linulus* (Thomas, 1910) (n=1). The Shannon index (H in Table 1) was 1.86 when the three seasons were taken into account, but varied according to season: 1.54 in June 2004, 1.66 in October 2004 and 1.83 in February 2005. Species richness and evenness indices were also calculated by season (Table 1).

In houses, the diversity and evenness indices were the lowest (H=0.99, E=0.61), because the commensal rodent community was mainly composed of *R. rattus* (68%, 28/41), which tends to evict the autochtonous species such as *M. erythroleucus*, *M. mattheyi*, *P. daltoni* and *H. simus* (Fig. 1). Conversely, cultivations were highly diversified (H=1.69), with 12 species more equally distributed than in houses (E=0.68) despite the predominance of *M. erythroleucus* (54%). In forests and orchards, the evenness index was highest (E=0.71) because the occurrence of *M. erythroleucus* decreased (42%), whereas those of *L. sikapusi* (30%) and *P. rostratus* (22%) increased. In savanna/fallow land, *M. erythroleucus* was highly representative (65%), with a significantly higher occurrence (p=0.02) than in forest/orchards. *L. sikapusi* and *P. rostratus* were not present in savanna/fallow land; however *U. ruddi*, *L. striatus*/*linulus* and *P. daltoni*, were present, so that a similar diversity index (H=1.28 and H=1.25) was evident in both habitats.

The seasonal variation in abundance and in reproduction is analysed below in detail for the four most common species, *M. erythroleucus* (46.4%), *L. sikapusi* (12.8%), *P. rostratus* (9.7%) and *R. rattus* (9.7%), and globally for the remaining ones.

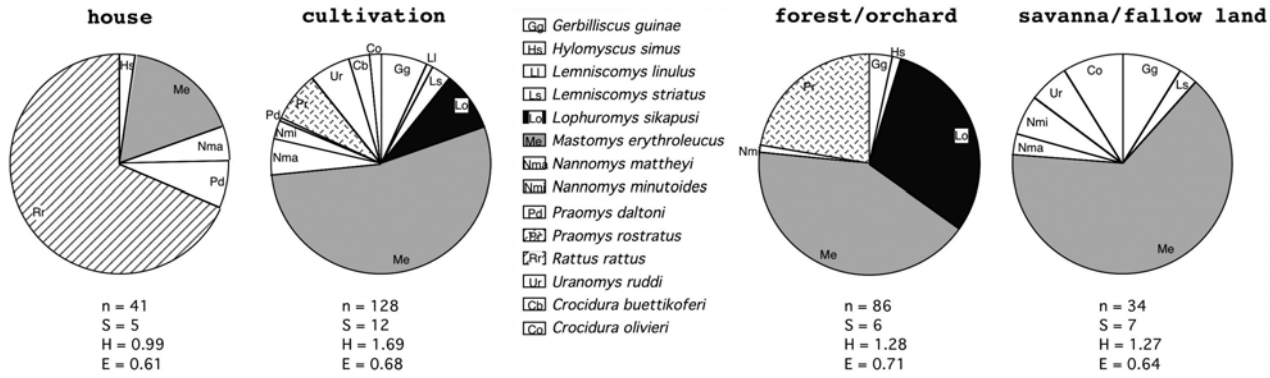

Fig. 1. – Distribution by habitat of the 14 species recorded at Gania. N, S, H and E correspond to sample size, species richness, species diversity and species evenness indices respectively (see material and method for definitions).

TABLE 1

Distribution of the small mammal community by season and by detailed habitat in Gania. Muridae: Gg=*Gerbilliscus* (*Tatera*) *guineae*, Hs=*Hylomyscus simus*, Ll=*Lemniscomys linulus*, Ls=*L. striatus*, Lo=*Lophuromys sikapusi*, Me=*Mastomys erythroleucus*, Nma=*Mus* (*Nannomys*) *mattheyi*, Nmi=*Mus* (*Nannomys*) *minutoides*, Pd=*Praomys* (*Myomys*) *daltoni*, Pr=*P. rostratus*, Rr=*Rattus rattus*, Ur=*Uranomys ruddi*; Soricidae: Cb=*Crocidura buettikoferi*, Co=*C. olivieri*. Sample size (N), species richness (S), diversity (H) and evenness (E) were calculated for each trapping session (see material and method for definitions). Italic values in brackets indicate the numbers of animals screened for Lassa virus.

Season	Houses	Nearby cultivations	Nearby forest/ orchard	Wooded fallow land	Grass-land	Remote cultiva-tions	Remote forest/ orchard	N	S	H	E
Early rainy (Jun 2004)	1 Me *(1)* 1 Nma (*1)* 5 Rr *(5)*	5 Gg *(5)* 3 Ls *(3)* 27 Me *(26)* 7 Nma *(6)* 3 Nmi *(3)* 6 Ur *(5)*	2 Gg *(2)* 1 Lo *(1)* 6 Me *(6)* 1 Nmi *(1)* 1 Pr *(1)*	1 Gg *(1)* 4 Me *(4)* 1 Nma *(1)* 2 Nmi *(2)*			9 Me *(9)*	**86**	**9**	**1.54**	**0.70**
Late rainy (Oct 2004)	1 Hs 5 Me 18 Rr *(3)*	1 Gg 8 Lo 28 Me *(2)* 1 Pd 2 Pr 1 Ur 2 Co	1 Gg 9 Lo *(1)* 7 Me 2 Pr *(1)*	7 Me 1 Ur	1 Ls 8 Me *(3)* 1 Ur *(1)* 2 Co *(1)*	1 Lo 1 Ls 2 Me 7 Pr 2 Cb	1 Lo 1 Me 8 Pr *(5)*	**129**	**11**	**1.66**	**0.69**
Dry (Feb 2005)	1 Me *(1)* 1 Nma 3 Pd 5 Rr	3 Gg 1 Ll 1 Lo 7 Me *(1)* 1 Ur 1 Cb	2 Lo 2 Me 3 Pr		2 Gg 3 Me 1 Co	1 Lo 5 Me *(2)* 1 Cb	1 Hs 13 Lo 11 Me *(1)* 5 Pr	**74**	**12**	**1.83**	**0.74**

TABLE 2

Mean trapping success (TS) and abundance index (AI) in % by species, habitat and season.

		mean TS			AI		
Species	**Habitat**	**Early rainy** (Jun 04)	**Late rainy** (Oct 04)	**Dry** (Feb 05)	**Early rainy** (Jun 04)	**Late rainy** (Oct 04)	**Dry** (Feb 05)
M. erythroleucus	Houses	0.3	1.7	0.3	0.6	2.2	0.5
	Cultivations	5.0	5.6	2.5	3.0	3.3	1.5
	Forest	5.0	4.4	4.3	3.0	2.7	2.6
	Savanna	6.7	12.5	5.0	4.0	7.5	3.0
L. sikapusi	Cultivations	0.0	1.7	0.4	0.0	1.0	0.3
	Forest	0.3	5.6	5.4	0.2	3.3	3.0
P. rostratus	Cultivations	0.0	1.7	0.0	0.0	1.0	0.0
	Forest	0.3	5.6	2.7	0.2	3.3	1.6
R. rattus	Houses	1.7	6.0	1.7	3.1	7.8	2.6

Spatio-temporal abundance and reproduction

Abundance index and mean trapping success by species and season are summarized in Table 2 in which June 2004, October 2004 and February 2005 correspond to early rainy, late rainy and dry season respectively.

Mastomys erythroleucus

The abundance index AI, analysed through an ANOVA, varied significantly by season ($p<0.0001$), *M. erythroleucus* being more abundant at the end of the rainy season (AI=4.4±1.9 ind/100m, n=53) than at the start (AI=3.0±0.3 ind/100m, n=46) or in the dry season (AI=2.2±0.6 ind/100m, n=28). AI also varied significantly by habitat ($p<0.0001$, Table 2), *M. erythroleucus* being more often trapped in the savanna/fallow land (AI=6.2±1.9 ind/100m, n=22) than in cultivations (AI=2.8±0.6 ind/100m, n=69) or in forest/orchards (AI=2.8±0.2 ind/100m, n=36). The two-way interaction "habitat x season" is significant ($p<0.0001$), suggesting that many rodents explored the savanna/fallow land at the end of the rainy season (Fig. 2). The age structure, based on eye lens weight ELW did not vary by habitat (ANOVA ns) but varied significantly by season ($p<0.0001$), revealing a younger population in the dry season (ELW=18.4±3.2mg, n=29), compared to the rainy season. Mean ELW values were similar in early (ELW=28.1±1.9mg, n=47) and late (ELW=27.9±9.9mg, n=58) rainy season, but the population sampled at the end of the rainy season (October 2004) was constituted by two cohorts, the old and the young, separated by only a few individuals in the intermediate ELW classes (Fig. 3). Reproduction, analysed through a multiple logistic regression, showed that sexual activity was significantly higher in the late rainy season ($p=0.0001$), than in the early rainy or the dry seasons. This analysis was made including sex and age (ELW) since these intrinsic variables are known to have a high influence on sexual activity ($p<0.0001$ for both). Here, high activity was mainly due to a higher rate in males (76%, 22/29) than in females (14%, 4/29), because the analysis in females alone did not show significant differences in sexual activity between the three trapping sessions. Habitat had no influence on sexual activity (Chi2 NS). In the dry season, the sex ratio was biased towards males (6 females vs 23 males). Litter size, here based on the number of foetuses, was 9.8 (range: 5-13, n=5).

Lophuromys sikapusi and *Praomys rostratus*

Season had a significant effect on abundance index in the two species ($p<0.0001$ for both), leading to higher abundances in the late rainy season (*L. sikapusi* and *P. rostratus*: AI=2.2±1.2 ind/100m, n=19) and in the dry season (*L. sikapusi*: AI=2.7±0.9 ind/100m, n=17; *P. rostratus*: AI=1.6ind/100m, n=8) than in the early rainy season (*L. sikapusi* and *P. rostratus*: AI=0.2ind/100m, n=1, Fig. 2). In the dry season, individuals of *L. sikapusi* were found deep in the forest (13/17 in remote forest/orchards), in contrast to the previous season where many of them were found in nearby cultivations and forest/orchards (17/19, Table 1). Habitat also had a significant effect ($p<0.0001$) for both species because these rodents were more abundant in forest/orchards (*L. sikapusi*: AI=3.0±0.6 ind/100m, n=26; *P. rostratus*: AI=2.4±1.0, n=19) than in cultivations (*L. sikapusi*: AI=0.9±0.3 ind/100m, n=11; *P. rostratus*: AI=1.0 ind/100m, n=9).

As for *M. erythroleucus*, the age structure in *L. sikapusi* varied seasonally ($p=0.01$), with the population older in the dry season (ELW=6.9±1.5mg, n=17) than in the late rainy season (ELW=5.5±1.9mg, n=19) (Fig. 3). At this period, sexual activity in both sexes was high (38%, 8/21) and then declined in the dry season (6%, 1/17). Conversely, the age structure in *P. rostratus* did not vary by season (ANOVA ns) and reproduction also continued in the dry season. Statistics for *L. sikapusi* and *P. rostratus* were based only on the samples from the late rainy and dry seasons, as the sample from the early rainy season was too small to be included (n=1 for each species in June 2004). The litter sizes were 3.5 (range: 3-4, n=4) in *L. sikapusi* and 4.1 (range: 3-5, n=7) in *P. rostratus*.

Rattus rattus

As for the rodents described above, the main commensal rodent, *R. rattus* was more abundant in houses in the late rainy season. Reproduction occurred during the rainy season and seemed to have ceased by the dry season. The sample sizes in June 2004 (n=5) and February 2005 (n=5) were too small to allow statistical analyses for abundance and age structure, and also to be sure of an absence of reproduction (Fig. 3).

Other species

Combining the two species of *Mus*, it is valuable to note that most of them (15/16) were trapped in the early rainy season. They were not detected in the other seasons, despite a similar trapping effort in the same habitats. Only one of 15 *M*us was sexually active in June 2004. In October 2004, two of two *G. guineae* (females), one of one *H. simus* (male), one of one *L. striatus* (male), one of one *P. daltoni* (female), three of three *U. ruddi* (one female, two males) and one of four *C. olivieri* (female) were sexually active. In February 2005, only three of five *G. guineae* (one female, two males) and one of one *H. simus* (female) were sexually active.

Detection of Lassa virus

In coastal Guinea, none of the animals was found to be infected with Lassa virus (Lecompte et al., 2006), nevertheless the specimens caught in Gania and examined for arenavirus infection are listed in Table 1 (for details in RT-PCR screening, see Vieth et al., 2007; Lecompte et al., 2006 and Fichet-Calvet et al., 2007). Of 289 small mammals collected, 106 were screened, distributed over all species and habitats.

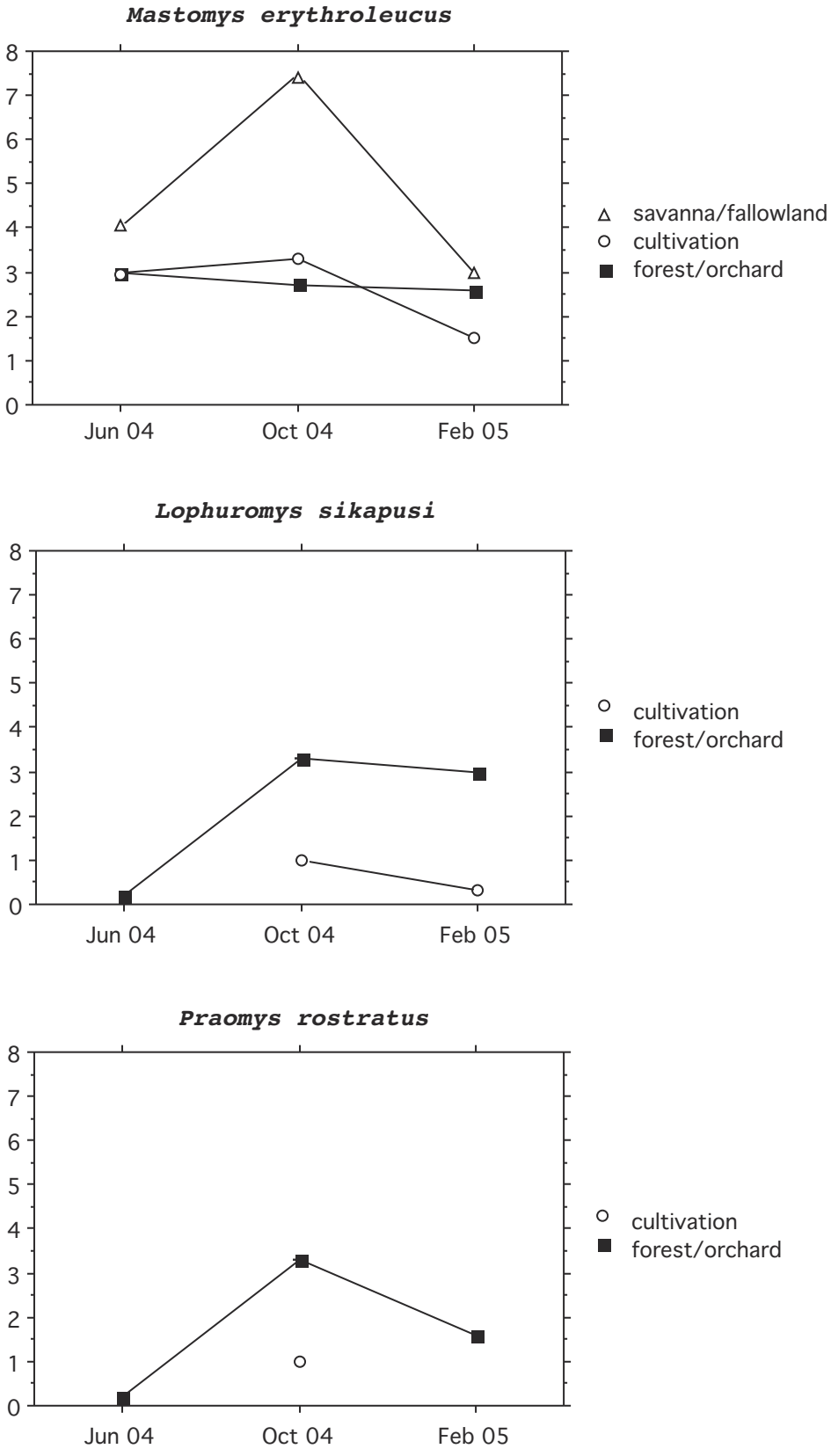

Fig. 2. – Abundance index (individuals/100m) in *Mastomys erythroleucus*, *Lophuromys sikapusi* and *Praomys rostratus* by habitat and by month of collection.

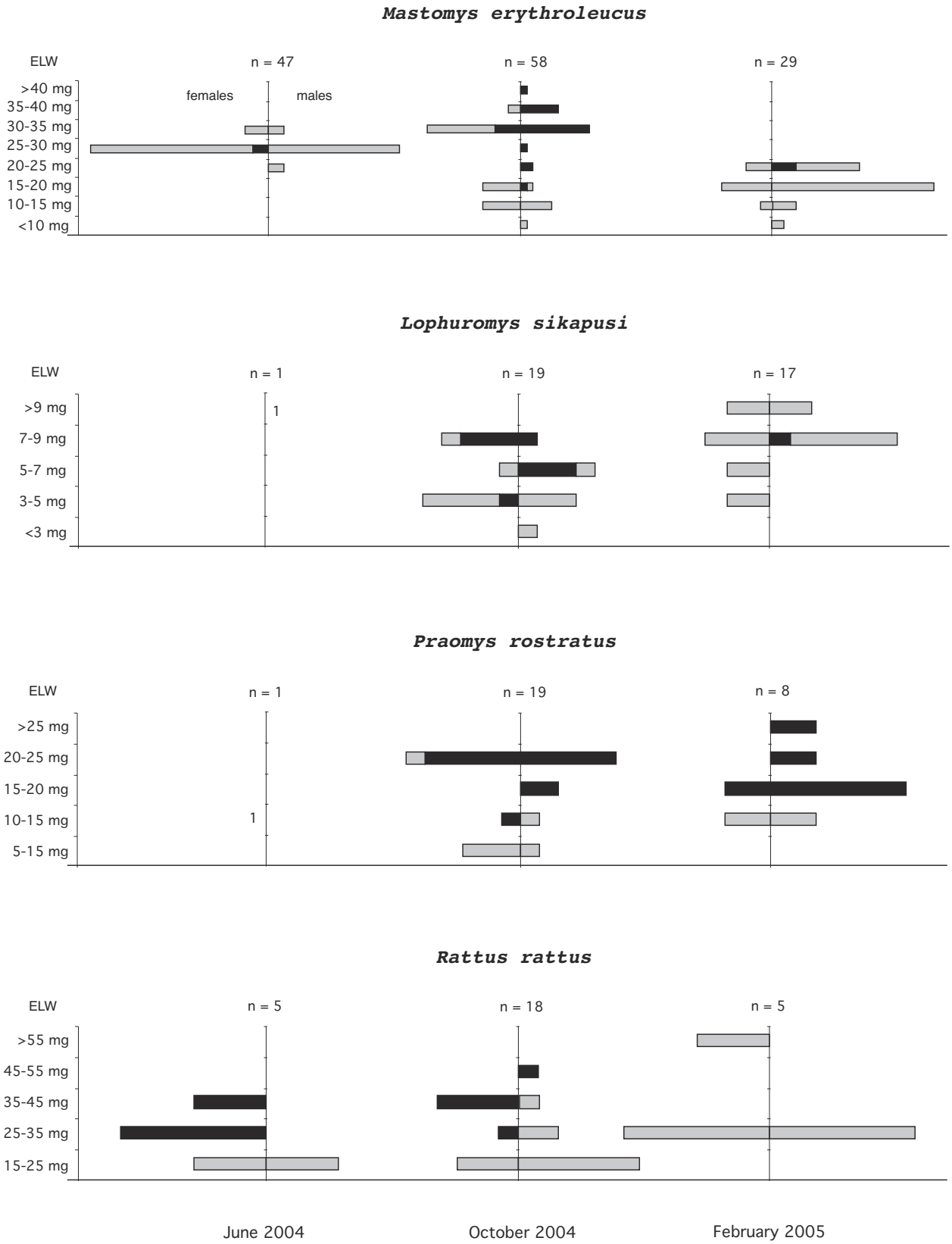

Fig. 3. – Distribution of sexual activity in *Mastomys erythroleucus*, *Lophuromys sikapusi*, *Praomys rostratus* and *Rattus rattus* by age (ELW: eye lens weight), sex and season. Each pyramid corresponds to the proportion of females (left) and males (right) sampled at each session (shaded area) in which the proportion of sexually active individuals is included (black area). To equalize the pyramid surface between small and large samples, the length of each horizontal bar reflects the proportion of individuals in each collection (total length of bars in each collection=1). n: sample size for each session.

DISCUSSION

The species richness, diversity and evenness indices (N=14, H=1.9 and E=0.7) were higher at Gania than at Kedougou, Senegal (N=12, H=1.5 and E=0.6 in BA, 2002). These studies are, nevertheless, comparable in time because several seasons were pooled, in space because trapping was performed inside and outside, and in methodology because the same kind of trap was used. Lower records of species by season in our study,– 10, 11 and 12 in early rainy, late rainy and dry seasons respectively –, than the 14 recorded globally, are probably a function of a cumulative effect from three trapping seasons instead of one only investigated in the other studies such as in Guinea or in Chad where species richness did not exceed 9 (GRANJON et al., 2004; DENYS et al., 2005). This suggests that studies on biodiversity should take into account the time scale for accurate estimations. Consequently, diversity in Gania, located between 12°W and 13°W in the transition zone (WHITE, 1983), but 280km south of Kedougou, indicates the Sudanian, Guinean and Congolian influences are remarkable for the combination of faunas from savannas and forests.

In our one-year survey, *M. erythroleucus* showed seasonal variations in abundance, the maximal abundance being in late rainy season. These variations were linked with the annual cycle in reproduction, which occurs principally during the rainy season, as was previously reported in Côte d'Ivoire, Senegal and Ethiopia (GAUTUN, 1975; HUBERT, 1982; DUPLANTIER et al., 1996; BEKELE & LEIRS, 1997). The young, born in the rainy season were able to breed in the season of their birth, and thus assisted in increasing population density, producing a double age pyramid in late rainy season (October). As the population structure was young in the dry season, February, with the presence of ±30 days old individuals (ELW correspondence in HUBERT & ADAM, 1975), we suspect that reproduction was continuing in the early dry season as well (November to January). Then, from late dry season (February) to early rainy season (June), reproduction probably ceased, as evidenced by the lack of young age classes in the early rainy season (June). Consequently, reproduction in *M. erythroleucus* would probably continue for eight months of the year, from June to January in Gania, whereas in Freetown, Sierra Leone, it was continuous throughout the year with a peak from May to January (BRAMBELL & DAVIS, 1941). The lower values recorded in February-April could be due to commensal individuals. In the western zone of Senegal, at Bandia, reproduction lasted five months, from July to November during the dry years ±300mm, but extended to seven months during the rainy years (±600mm) (HUBERT, 1982). The timing of reproduction is then, largely flexible in this species, according to climatic conditions and underlying food availability.

The abundance index analysed here by habitat showed higher values in savanna/fallow land (3 to 7.5 ind/100m) than in nearby cultivations (1.5 to 3.3 ind/100m), suggesting that savanna/fallow land could serve as a source for rodent dispersal into cultivations. In Gania, *M. erythroleucus* also inhabits the forest/orchards, remaining stable through the year (2.6 to 3.0 ind/100m). Complementary to studies in Senegal and Guinea (BA, 2002; DENYS et al., 2005), we show here that *M. erythroleucus* is well adapted to live in the wooded habitats, sharing space with *L. sikapusi* and *P. rostratus*.

The latter species occurred in many trap lines at Gania, probably because of a well-preserved forest. *L. sikapusi* was more abundant in October 2004 and February 2005 than in June 2004. Such variations have already been observed in Gambari forest, Nigeria, where these rodents were abundant during one of three studied years (HAPPOLD, 1977). In our study, *L. sikapusi* was also more abundant than in primary rainforest such as in Gambari forest or in Makokou forest in Gabon (DUPLANTIER, 1989), and also occurred in cultivations, which offered at the end of the rainy season a dense herbaceous coverage made by cucumbers and sweet potatoes. Our observations in Guinea confirm the preference of this species towards the habitats of secondary forest and moist cultivations. Reproduction seems to decrease (1/17) in the dry season, as was also observed in Nigeria (HAPPOLD, 1977).

The systematics of *Praomys* was recently amended from molecular studies (LECOMPTE et al., 2002b; NICOLAS et al., 2005; NICOLAS et al., 2008). As no data are available for *P. rostratus* dynamics, we here compare our results to those available for closely related species *P. tullbergi* and *P. misonnei* (formely *tullbergi*) (NICOLAS et al., 2005). In Guinea, this species occurred in secondary forests mixed with orchards, hedgerows and cultivations, and also in semi-evergreen forest such as the Park of Upper Niger (ZIEGLER et al., 2002). As for *L. sikapusi*, its abundance was higher in forest habitats (0.2 to 3.3 ind/100m) than in cultivations (0 to1 ind/100m), but never reached the level of densities of *P. tullbergi* in primary forests in Nigeria (15 to 20/ha in HAPPOLD, 1977), or of *P. misonnei* in Gabon (4 to 20/ha in DUPLANTIER, 1989). Here, reproduction persisted in the dry season, similarly to observations made in Côte d'Ivoire (LIM & COEVERDEN DE GROOT, 1997), in Nigeria for *P. tullbergi* (HAPPOLD, 1978), and in Gabon for *P. misonnei* (NICOLAS & COLYN, 2003).

Pygmy mice, *Mus* spp, were either numerous, or were not caught at the other times. This binary variation "all or nothing" has already been recorded by MONADJEM, 1999 in Swaziland, where *M. minutoides* was captured in relatively large numbers in winter, and then almost completely disappeared from samples in summer and autumn. In agreement with this author, we speculate that *Mus* spp. did not "disappear" from the study area but simply stopped visiting traps in the late rainy season and dry season when natural food was abundant. As for many species that were sexually active, the late rainy season also corresponds to the peak reproductive season, leading many species to move for mating, breeding and dispersing, which could cause the pygmy mice to not enter the traps. Nevertheless, their abundance at the start of the rainy season is congruent with the longitudinal survey realised on the "Leggada" populations (the former name of *Mus* (*Nannomys*)) in Côte d'Ivoire in 1969-1971 (BELLIER, 1974). That study noted increased numbers in June and July, subsequent to the recruitment of young between February and April. Moreover, the systematics of *Mus* (*Nannomys*) species is unsatisfactory owing to the lack of discriminating morphological characters, and it is further

confounded by the sympatric coexistence of sibling species (JOTTERAND-BELLOMO, 1988; VEYRUNES et al., 2004). Here, we found two species in a small sample, *M. matheyi* and *M. minutoides* even being trapped on the same trap lines; these results suggest the need for caution in interpretation of previous studies on *Mus* (those without a molecular or karyotypic identification), as an amalgam of these morphologically-similar species is strongly suspected.

The houses in Gania had a surprisingly low infestation of rodents in the dry season (mean TS for all species =3.3%), regardless of their trend to migrate inside at this season. Higher levels of infestation have been observed in other study sites in Senegal and Guinea (BA, 2002, FICHET-CALVET et al., 2007). This could be due to rodent management, regularly initiated by villagers, but also to the absence of bush fires, which were forbidden in Gania village. Such management could preserve seeds and shelters for rodents in natural habitats, hence removing the necessity to enter houses for food and shelter. Preservation of natural habitats such as savanna and forest, and anthropized ones, such as wooded fallow land, may allow a high species diversity, in contrast to places where farmland is burned (PAPILLON et al., 2006).

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The effects of a meridic diet on the sex ratio of offspring, on glycogen and protein content, and on productivity and longevity of adult *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) for five generations

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ABSTRACT. In this study, we determined the effects of a meridic diet on adult emergence, glycogen and protein level of the endoparasitoid *Pimpla turionellae*, and investigated female lifetime, adult emergence rate, egg numbers and hatching for five generations. The meridic and control diets were fed to *P. turionellae* for 28 days. The meridic diet was associated with a significant increase in the total adult emergence, which reached 93.33% on day 16; 100% females were produced from eggs laid on day 25. The meridic diet significantly decreased glycogen level in *P. turionellae* on days 13 and 16, while it significantly increased the protein level on days 13, 16 and 19 in comparison to the control natural diet. The meridic diet did not negatively affect total adult emergence, lifetime and egg numbers over the first and second generations when compared to the control. However, female lifetime and egg numbers were significantly decreased in F3, F4 and F5 generations. This manuscript is the first to report rearing the adult stages of *P. turionellae* on meridic diets for five generations. It shows that a chemically-defined meridic diet may be beneficial in improving the biological and biochemical fitness of the first couple of generations of these parasitoids reared for use in biological control programs.

KEY WORDS : *Pimpla turionellae*, sex ratio, synthetic diet, glycogen, protein, adult emergence.

INTRODUCTION

Chemical pest management has been widely used, even though it can have a significant negative effect on the ecosystem. Chemicals affect not only the target pests, but also collaterally damage beneficial insects such as predators, parasitoids, and pathogens (SINGH, 1977).

Species of Hymenoptera are still widely used in biological pest control, and are easy to rear in *in vitro* culture by using an artificial diet (YAZGAN, 1981; EMRE, 1988). Entomophagous parasitic hymenoptera have a long life cycle, and the nutrients required for their survival and reproduction, such as proteins, lipids, carbohydrates, vitamins and inorganic salts, are supplied from plant juice, pollen and host haemolymph (LEUIS, 1961).

One of the main problems for continuous mass culturing of parasitic hymenopterans is obtaining the required hosts. Theoretical and empirical evidence suggest that according to the qualitative and quantitative combinations of the food source, the stored glycogen and protein play a key role in affecting egg number, emergence ratio, and transition time to adulthood (DADD, 1985; CANGUSSU & ZUCOLOTO, 1997). For a successful and efficient mass production system, egg number and sex ratio are important (ORR & BOETHEL, 1990; RAMADAN et al., 1995), and work on the latter has often been successful (WERREN, 1987; WEST et al., 2002).

In biological control, the target populations of pests should be kept at an acceptable level, and at the same time, side effects on the ecosystem should be minimized; thus a sufficient number of biological control agents must be introduced to the area where the pests species are.

Because parasitoid hymenopterans can determine the sex of the adult insect by controlling the sperm entrance to the egg with their haplo-diploid sex determination (FLANDERS, 1956), they are the ideal organisms for sex ratio studies (GODFRAY, 1994). The sex ratio can be affected by host size, sperm morphology, coupling rate, temperature, photoperiod, and qualitative and quantitative nutritional requirements (WILKES, 1964; HOLSCHER & VINSON, 1971; SANDLAND, 1979; HAGLEY & BARBER, 1992; ALLEN et al., 1994; KAZMER & LUCK, 1995; COŞKUN et al., 2005). As well as keeping the sex ratio at the optimum level, it is important to ensure diets and ambient conditions appropriate to the insect species (ETZEL & LEGNER, 1999).

The ichneumonid endoparasitoid *Pimpla turionellae* L. is used in biological control against numerous species of Lepidoptera, including the larvae or pupae of the black-veined white *Aporia crataegi* (L) (Pieridae), the gypsy moth, *Lymantria dispar* (L) (Lasiocampidae), and the Mediterranean flour moth, *Ephestia kuehniella* Zell. (Pyralidae) (THOMPSON, 1957). The ichneumonid lays its eggs in the lepidopteran species, thus assuring the continuation of its population. The effects of changing the synthetic diet developed by EMRE (1988), or of adding nutrients, on egg number, hatching, lifespan, sex ratio, glycogen and protein amount in the insect, have been documented only for the first generation (ÖZALP & EMRE, 2001; SULANÇ & EMRE, 2000; BAYKAL et al., 2005; COŞKUN et al., 2005).

It is important to study the effects of changes in reproduction and development on subsequent generations for mass rearing programs. In the present study, we quantified the effect of a meridic diet on *P. turionellae*, focusing on sex ratio, glycogen and protein levels in the first generation, and on sex ratio, egg number laid by adult females, hatchability, and lifespan for five generations.

TABLE 1

Composition of the chemically-defined synthetic diet fed to *P. turionellae* adults (EMRE, 1988)

Constituent	mg/100ml diet	Constituent	mg/100ml diet
L-Amino acid mixture	3000.00	Water soluble vitamin mixture	284.38
Alanine	210.00	Ascorbic acid	10.6105
Arginine-HCl	150.00	Biotin	0.0379
Aspartic acid	195.00	Ca-Panthothenate	2.8042
Cysteine	39.00	Choline chloride	246.3158
Glutamic acid	315.00	Folic acid	0.1137
Glycine	192.00	Inositol	17.0526
Histidine	120.00	Nicotinic acid	5.6842
Hydroxproline	57.00	Pyridoxine-HCl	0.2842
Isoleucine	156.00	Riboflavin	1.3263
Leucine	231.00	Thiamine-HCl	0.1516
Lysine	159.00		
Methionine	90.00	Inorganic salt mixture	75.00
Phenylalanine	165.00	$FeCl_3$ $6H_20$	2.1583
Proline	246.00	K_2HPO_4	45.0129
Serine	195.00	Na_2HPO_4 $12H_2O$	6.2201
Threonine	165.00	$MgSO_4$ $7H_2O$	15.7853
Tryptophane	60.00	$MnSO_4$ H_2O	0.0479
Tyrosine	120.00	$CoCl_3$ $6H_2O$	0.5798
Valine	135.00	$CuSO_4$ $5H_2O$	0.6721
		$CaCl_2$	3.6684
Lipid mixture	540.96	$ZnCl_2$	0.8552
Cholesterol	138.8430		
Linoleic acid	8.0331	Miscellaneous	
Linolenic acid	25.5537	Ribonucleic acid	75.00
Oleic acid	10.5950	Sucrose	14000.00
Palmitic acid	0.6777	2N KOH	280.00
Stearic acid	0.2314	2N K_2HPO_4*	14.03
Tween 80	357.0248	Distilled water to 100 ml	

*: Added into the water soluble mixture solution

MATERIALS AND METHODS

Maintenance of adult wasps

Wasps of *P. turionellae* were reared in the laboratory on the pupae of the greater wax moth, *Galleria mellonella* (L) (Lepidoptera: Pyralidae), and fed a diet of 50% honey solution, *G. mellonella* pupae and synthetic diet (EMRE, 1988). The composition of the chemically-defined synthetic diet, consisting of amino acids, lipids, vitamins, inorganic salts, sucrose and other nutrients, is shown in Table 1. To prepare the synthetic diet, the L-amino acid mixture, inorganic salt mixture, ribonucleic acid and sucrose, were dissolved in 50ml 90ºC distilled water. The solution was allowed to cool, the lipid and vitamin mixtures added, and the pH of the diet adjusted to pH 6.5 with 2N KOH, and finally the volume was brought to 100ml with distilled water.

For the experiments, we used recently matured *P. turionellae,* which had not been fed or mated. They were held in a cage approximately 20x25x25cm. The experimental group then received a liquid synthetic diet at regular intervals on 3x3cm aluminium foils that were put into the bottom of the cages. The control group received equal parts liquid honey and water, absorbed on a piece of cotton, along with five *G. mellonella* pupae every three days; the pupae were kept in the cage for an hour and then removed. This was repeated every three days at the same time throughout the experiment.

Determination of sex ratio

The method that was used to evaluate the effect of the meridic diet on *P. turionellae* sex ratio is described by COŞKUN et al. (2005). Ten females and five males that matured on the same day, were transferred to each experimental cage. Simultaneously, 10 *G. mellonella* pupae were provided for 1 hour, in which the wasps laid their eggs. After oviposition, all pupae were removed, placed in a beaker, and held until the adults emerged. The number of emerging male and female wasps was recorded, and pupae were dissected to determine the number that failed to produce an adult wasp. The female emergence ratio is the number of females emerging compared to the total number of emerging adults. This experiment was repeated three times and data were pooled for statistical analysis.

Adult emergence

Adult emergence was determined by calculating, as a percentage, the number of emerged individuals compared to the total number of pupae that were placed in the cages to be parasitized.

Determination of glycogen and protein levels

Four females, on each day, were placed in a 1000ml beaker covered with a highly porous cloth. The insects were removed to evaluate the synthesis of glycogen and protein beginning 10 days after the start of the experiment and then every three days until day 28. The anthrone test was used for determination of glycogen, and the quantitative Biuret test was used for determination of protein (ROE et al., 1961; PLUMMER, 1971).

For the protein and glycogen extraction, insects were placed in 10% trichloroacetic acid (TCA) solution and homogenized for five minutes. The homogenate was centrifuged at 3500g for 15min. Total proteins were determined spectrophotometrically at a wavelength 540nm. In order to determinate the total glycogen, aliquots of the supernatant were mixed with 96% ethyl alcohol for 24h at 37ºC, and then centrifuged at 3500g for 30 minutes. Total

glycogen of the pellet was determined spectrophotometrically at a wavelength 620nm.

All of the experiments were repeated at least three times under laboratory conditions at 24±2ºC, 75±5% humidity and 12 hours of light photoperiod. The statistical analyses were done with "Independent Samples Test" (T test). Differences between groups were considered to be significant at $p \leq 0.05$

Rearing adult *P. turionellae* for five generations with a meridic diet

Experiments were conducted on five successive generations to determine the effects of a meridic diet on (i) female emergence, (ii) number of eggs produced per female during her lifespan, (iii) the percentage of eggs hatched during a lifespan (hatchability), and (iv) the average female lifetime corresponding to each generation (lifetime). Generations were named as F1, F2, F3, F4, and F5.

To evaluate the effect of meridic diet on sex ratio and adult emergence in each generation, 10 female and 5 male were placed in cages and until the insects were 28-days-old data were gathered as described above for one generation.

To determine the effect of the meridic diet on egg number, hatchability and lifetime in each generation, four unfed and unmated females and two males were placed into a 1000cc beaker which was then closed by cheesecloth. The females were allowed to parasitize *G. mellonella* pupae after 10 days and at subsequent three day intervals until day 28. The provision of pupae for parasitism took about 30min, and at the end of this period, pupae were collected from the beakers and held for 24 hours for embryonic development. Pupae were then dissected in a petri dish with 0.8% NaCl, and the eggs gently transferred into another petri dish with 0.8% NaCl. After 24h incubation, numbers of laid and hatched eggs were counted under a stereomicroscope. In all replicates (3), the mean numbers of eggs per female were calculated by dividing the total number of eggs by the number of females. Hatchability was calculated by dividing the hatched eggs by the total number of eggs laid.

To calculate the lifetime of the female *P. turionellae*, feeding was continued until all insects died. The cages were checked daily for mortality, and after all females died, the average longevity of wasps was determined for each generation. The corresponding procedure was repeated for the control group.

Statistical analyses

Data from the effect of the meridic diet over the five generations were compared with the control. Statistical analysis was done with "Student-Newman Keuls Test (SNK)" (SOKAL & ROHLF, 1969). Differences between groups were considered to be significant at a probability level of $p<0.05$%.

TABLE 2

Effects of meridic diet on the sex ratio of *P. turionellae*

	Adult Emergence (%)	
	Total	Female
Diets	Mean±S.D *	Mean±S.D *
Meridic diet	74.28±0.82 a	72.34±0.72 a
Natural (control)	68.57±1.43 b	67.36±0.12 b

*Values followed by the same letter are not significantly different from each other (P<0.05, T test)

TABLE 3

Effects of meridic diet on the emergence ratio of *P. turionellae* according to days

	Emergence adult (%)			
Day eggs laid	Total		Female	
	Meridic diet Mean±S.D *	Control diet Mean±S.D *	Meridic diet Mean±S.D *	Control diet Mean±S.D *
10	70.00±5.77 a	66.67±3.33 a	45.79±2.85 a	39.57±3.29 a
13	83.33±3.33 a	53.33±3.33 b	68.05±3.67 b	78.25±3.55 a
16	93.33±3.33 a	80.00±5.77 b	67.78±1.11 b	74.74±1.84 a
19	76.67±3.33 a	66.66±3.33 b	73.81±1.19 b	84.91±0.79 a
22	73.33±3.33 a	76.67±3.33 a	72.62±1.19 a	60.71±1.78 b
25	76.67±3.33 a	70.00±0.00 a	100.00±0.00 a	71.43±0.00 b
28	46.67±3.33 a	60.00±5.77 a	78.33±1.66 a	61.27±2.82 b

* Values followed by the same letter in the same line are not significantly different from each other (P<0.05, T test)

TABLE 4

Effects of meridic diet on total protein and glycogen levels of adult *P. turionellae*

	Average Weight of insect (g)		Total protein (%)		Total glycogen (%)	
Day eggs laid	Meridic diet	Control diet	Meridic diet Mean±S.D*	Control diet Mean±S.D*	Meridic diet Mean±S.D*	Control diet Mean±S.D*
10	0.0252	0.0317	5.45±0.03 a	5.50±0.03 a	0.090±0.03 a	0.095±0.04 a
13	0.0241	0.0319	5.91± 0.04 b	5.50±0.02 b	0.130±0.06 a	0.290±0.03 b
16	0.0261	0.0311	6.30±0.02 b	4.90±0.02 b	0.129±0.03 a	0.310±0.03 b
19	0.0278	0.0289	6.94±0.02 b	6.44±0.02 b	0.140±0.05 a	0.165±0.04 a
22	0.0309	0.0284	7.25±0.03 a	7.30±0.01 a	0.158±0.06 a	0.162±0.05 a
25	0.0321	0.0235	6.20±0.02 a	6.10±0.02 a	0.169±0.04 a	0.153±0.06 a
28	0.0327	0.0212	5.96±0.01 a	6.00±0.02 a	0.185±0.12 a	0.129±0.05 a

* Values followed by the same letter across the same line are not significantly different from each other (P<0.05, T test)

RESULTS

Overall, total adult emergence (74%) and female emergence (73%) were significantly higher in wasps fed the experimental meridic diet than in wasps fed the control diet (68% and 67%) (Table 2).

When considered over time, total adult emergence was significantly higher in the experimental meridic diet group than in the control group from eggs deposited on days 13, 16 and 19 (Table 3); there were no significant differences on the other days. Maximum adult emergence (93.33%) occurred from eggs deposited on day 16 in the experimental group fed the meridic diet.

Although female emergence from eggs deposited on days 13, 16 and 19 was significantly lower in the experimental meridic diet group than in the control group, it was significantly higher on days 22, 25 and 28 (Table 3). Maximum female emergence (100%) occurred in the experimental group on day 25.

The effects of meridic and natural diets on the protein and glycogen levels of *P. turionellae* are shown in Table 4. The total percentage of protein in wasps fed the experimental meridic diet was significantly increased, on days 13, 16 and 19, compared to wasps that received the control diet. Glycogen levels were not significantly different between the wasps receiving the meridic and control diets for most of the study period, the exception being on days 13 and 16 when levels were significantly lower in the meridic diet groups compared to the control.

The effect of a meridic diet on total adult emergence of *P. turionellae* over the five generations is shown in Table 5. For wasps fed a meridic diet, there was a significant decrease in total adult emergence in the F4 and F5 generations compared to the preceding generations, and compared to the control. Maximum adult individual emergence occurred in the first generation. In percentage female emergence, a significant decrease was observed only in the F5 generation compared to the preceding generations, and compared to the control.

The effects of a meridic on the total egg numbers, hatchability and the lifetime of the female of *P. turionellae* over the five generation are shown in Table 6. The female lifetime was significantly decreased in the F3 (34 days), F4 (32 days) and F5 (32 days) generations compared with preceding generations (F1, 43 days; F2, 41 days) and with the control (45 days). The number of eggs per female was significantly higher in F1 and F2 generations (41 and 38 respectively), than in subsequent generations (F3, 29; F4, 25; F5, 24), but was not significantly different from the control. In percentage hatchability there were no significant differences between the five generations, or between any of the generations on the meridic diet and control wasps.

TABLE 5

Effects of meridic diet on Adult Emergence of *P. turionellae* over five generation

	Adult Emergence (%)	
Generation	**Total Mean±S.D ***	**Female Mean±S.D ***
Control	71.42±2.47 ab	69.89±1.85 a
F1	83.33±3.71 a	77.05±1.15 a
F2	72.38±1.71 b	70.31±4.20 a
F3	78.09±1.26 b	77.40±0.97 a
F4	57.08±2.53 c	73.75±2.10 a
F5	55.00±1.44 c	59.96±2.85 b

* Statistical analyses were done separately for each generation and for the control group. Values followed by the same letter are not significantly different from each other, P<0.05

TABLE 6

Effects of meridic diet on lifetime, egg number and fertility of adult female *P. turionellae* over five generations

	Survival (Days)	Eggs Number (No of egg/ female)	Hatchability (%)
Generation	**Mean±S.D ***	**Mean±S.D ***	**Mean±S.D ***
Control	44.56±1.10 a	36.14±1.58 a	82.66±1.74 a
F1	43.44±1.11 a	41.39±3.23 a	81.14±2.50 a
F2	41.18±1.00 a	37.67±0.46 a	76.03±0.78 a
F3	34.42±1.04 b	28.69±1.55 b	80.99±4.71 a
F4	32.46±1.76 b	24.86±3.36 b	80.44±0.65 a
F5	32.36±0.49 b	24.25±0.52 b	80.54±2.69 a

* Statistical analyses were done separately for each generation and for the control group. Values followed by the same letter are not significantly different from each other, P<0.05

DISCUSSION

The main findings of this study are that the meridic diet significantly increased the sex ratio of *P. turionellae* in favour of females, and that it also significantly increased the protein content of the individuals. Thus this study improves our understanding of the role of synthetic diets in maintenance of insects required for biological control. It is also the first study to report rearing the adult stages of *P. turionellae* on meridic diets for five generations.

The growth, development, reproduction, and behaviour of insects are closely related to the quantity and quality of their food, and it is crucial that dietary components are well balanced (PARRA, 1991; IDRIS & GRAFIUS, 1997; CHANG, 2004; MAGRO et al., 2006). The nutrients that larvae receive affect not only the growth rate, development, weight, and survival of the larvae, but also the fitness of the adult (PARRA, 1991).

Our study shows that the meridic diet increased both total and adult female emergence ratios. Diet has previously been shown to affect the reproductive performance of *P. turionellae* (EMRE, 1988), and in *Itoplectis conquistor,* increase in amino acid amount in an artificial diet was related to an increase in male emergence (YAZGAN, 1972).

A decrease in adult emergence (evident in our study on both the control and meridic diets) is an unavoidable result of aging. However, in our study the meridic diet did eliminate the effect of aging on female emergence between days 22 and 28.

Protein and glycogen are used as the main energy sources in many insect species. Proteins directly affect the reproductive performance of the insects (DADD, 1985),

and CANGUSSU & ZUCOLOTO, (1997) showed that the amount of protein that insects store affects egg number, adult emergence and adult size. In our study of *P. turionellae* we showed that protein and glycogen levels may correlate with adult emergence.

In many insect species, the relationship between available energy reserves and coupling success and stability in reproductive behaviour is very important (PETERSSON, 1989). In our study the meridic diet decreased the glycogen levels on days 13 and 16 compared to the control; this could be a result of nutritional stress while the insects were adapting to the meridic diet, and glycogen from their own reserves was required to compensate for it.

Considerable amounts of glycogen are needed by many adult insects for flying, movement, searching for hosts and parasitizing them. For example, *Anopheles freeborni*, consumed over 50% of their current energy reserves for a flight of 40 minutes. Efficient restocking of energy reserves therefore may contribute to mating success (YUVAL et al., 1994). Glycogen reserves of insects change according to age, temperature, rearing and photoperiod. It is known that there is a significant relationship between the change of glycogen amount based on age, diet and mating (YUVAL et al., 1994). Levels of total sugars and glycogen in sugar-fed flies are positively correlated with wing length, possibly indicating higher accumulation of storage sugars by larger flies. These results are generally in agreement with previous reports on nutrient levels in *Pseudacteon tricuspis* (FADAMIRO & CHEN, 2005; FADAMIRO et al., 2005).

In our study, a lower female individual emergence rate on the meridic diet for days 13 and 16 was correlated with lower glycogen levels than the controls on those days. This is similar to the decrease in egg numbers associated with a decrease in glycogen levels reported for *P. turionellae* by ŞEKER & YANIKOĞLU (1999).

Amino acids are essential for the growth and development of insects (CHEN, 1985), and many researchers stress their key role in the reproduction of parasitoids under laboratory conditions (THOMPSON, 1983; VINSON, 1994; NETTLES, 1987; HU et al., 1998). The effect of different concentrations of amino acids on insect development has been extensively studied (FRIEND et al., 1957; CANGUSSU & ZUCOLOTO, 1997; CHANG, 2004). The differences in egg numbers in insects such as *Dacus olea* (TSIROPOULOS, 1980), *Acheta domesticus* (MCFARLANE, 1988), and *Melanogryllus desertus* (BASHAN & BALCI, 1994) depending on diet, illustrates the importance of the nutritional balance on the reproduction period of *P. turionellae.*

The data obtained from generations F1 to F5 show that there is a linear relationship between lifetime and egg number. The fact that longer survival in the control, F1 and F2 generations resulted in greater total egg numbers produced, shows that there is a requirement for diets that prolong the lifetime as much as possible. Previous studies have shown that diet, particularly carbohydrate, affects insect lifetime (ÖZALP & EMRE, 2001; JACOB & EVANS, 2004; ONAGBOLA et al., 2007). In the synthetic diet we used for *P. turionellae,* sucrose, which is a strong phagostimulant, was used as a carbohydrate source because ÖZALP & EMRE (2001) had shown sucrose to be the most beneficial among the 23 different carbohydrates tested for their effect on *P. turionellae* lifetime. We suggest that future studies should determine the optimal amount of sucrose, as the carbohydrate source, in meridic diets for *P. turionellae*.

We observed that although there was no decrease of egg number, female emergence rate or lifetime for the first two of five generations, there were decreases in the fourth and fifth generations compared to the control. We suggest that adding vitamin E to the diet may rectify these problems, since EMRE & YAZGAN (1990) observed that 0.0010% vitamin E in the meridic diet increased egg number, and COŞKUN et al. (2005) observed that adding 0.0010 and 0.0015% vitamin E markedly increased female individual emergence.

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Comparative myology of Leiosauridae (Squamata) and its bearing on their phylogenetic relationships

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ABSTRACT. We present a study of the musculature of the leiosaurids, an ecologically diverse family of lizards that inhabits southern South America. Our first goal is to contribute to a better understanding of the anatomical structures, and particularly the muscular features, of leiosaurids and the related polychrotids *Anolis* sp. and *Polychrus* sp. To study these myological features in a cladistic context, we added 162 new cranial and postcranial myological characters to the 82 morphological characters of FROST et al. (2001) and assembled a matrix including 20 taxa and 244 characters including all leiosaurid genera, and analyzed them cladistically (data set II). We combined and contrasted our own muscular data with the morphological data of FROST et al. (2001) in different data sets (I, II, III) in order to analyze the evidence provided by myology against that provided by osteological and external features. The Enyaliinae is paraphyletic in all our analysis. In our analyses of data sets II and III, the Leiosauridae appears as a monophyletic group. We recovered Leiosaurinae as monophyletic in the analysis of data set II, III, and in the supertree. *Leiosaurus* genus is monophyletic in all our analyses, except that based on our data set I. *Diplolaemus* genus is monophyletic in all our analyses. *Pristidactylus* genus is a clade in our analyses of data sets II and III, while *Enyalius* genus appears as monophyletic in our analyses of data sets I, II and III. *Anisolepis* and *Urostrophus* genera are monophyletic in our supertree.

KEY WORDS : muscles, Maximun Parsimony, *Anolis*, *Polychrus*, Squamata

INTRODUCTION

The Leiosauridae (FROST et al., 2001) is an ecologically diverse group of South American lizards including arboreal taxa such as *Enyalius* sp. (Wagler, 1830) and *Urostrophus* sp. (Duméril & Bibron, 1837), and terrestrial ones, such as the pristidactylines. FROST et al. (2001) consider the Leiosauridae to be composed of the Leiosaurinae and Enyaliinae (but see SCHULTE et al., 2003). Leiosaurines (*Diplolaemus* sp. (Werner, 1898), *Leiosaurus* sp. (Duméril & Bibron, 1837) and *Pristidactylus* sp. (Fitzinger, 1843)) occur mainly in Argentina, although some *Pristidactylus* species are endemic to Chile. Enyaliines *(Anisolepis* Mocquard 1887*, Enyalius, Urostrophus)* are also found in Argentina, but *Enyalius* sp. inhabits mainly Brazilian regions. The taxonomy of the Argentinian species of leiosaurids was studied by GALLARDO (1961; 1964), DONOSO-BARROS & CEI (1969), CEI (1986), ETHERIDGE & WILLIAMS (1991), and more recently CEI et al. (2001; 2003), among others. Chilean species were partially examined by DONOSO-BARROS (1975), but leiosaurids remain less studied than any other iguanian lizards. The relatively small and slender lizards of the family Polychrotidae (sensu FROST & ETHERIDGE, 1989; subfamily Polychrinae sensu SCHULTE et al., 1998) are usually regarded as being closely related to the leiosaurids (FROST et al., 2001; CONRAD, 2008).

Morphological traits of leiosaurids were explored by FROST et al. (2001) in their phylogenetic analysis of the iguanian lizards. These authors included a total of 82 anatomical features in the analysis, which thus remains the most comprehensive cladistic study of leiosaurids, and thus is used as the phylogenetic framework for this study. However, leiosaurid myological structures were not included in that analysis. The scarcity of information on myology imposes serious limitations on the effective discussion of the functional anatomy, ecomorphology, phylogeny and evolution of this ecologically diverse group of lizards.

One of the main goals of this paper is to increase the understanding of the anatomy, and particularly of the myology, of leiosaurids. Another goal is to examine the bearing of myological characters on leiosaurid phylogenetic relationships in a broader anatomical and evolutionary context. We add 162 new cranial and postcranial myological characters to those 82 already analyzed by FROST et al. (2001), and assemble a matrix of 48 taxa and 244 characters (resulting in the largest morphological data set published so far for this group of lizards). We also discuss certain myological features that were found in some of the taxa examined (e.g. *Urostrophus* sp.) and that have not been previously recorded.

MATERIALS AND METHODS

We studied 75 specimens representing 16 leiosaurid species (including all genera and 50% of the described species insofar), 16 polychrotid species, and one corytophanid species (Appendix 1). All voucher specimens are deposited in the collection of the Instituto de Herpetología, Fundación Miguel Lillo, Tucumán, Argentina. Macroscopic observation of muscles was performed

using a binocular dissection microscope. The examined specimens include all of the leiosaurid genera of the FROST et al. (2001) analysis. Because of their scarcity in collections, it is difficult to obtain leiosaurid specimens for dissection; therefore the number of species we were able to study was constrained. However, whenever possible we also included morphological data obtained by other authors in our analysis, such as that provided by CEI et al. (2003) regarding *Diplolaemus sexcinctus* (Cei et al., 2003) and by CEI et al. (2001) concerning *Pristidactylus nigroiugulus* (Cei, Scolaro & Videla, 2001). We conducted two analyses: 1) one using only our 162 myological characters (data set I: 20 taxa x 162 characters), using *Polychrus* sp. (Cuvier, 1817) as an outgroup (Appendix 2; a detailed list of these myological characters, including hind-limb features that were not published previously, is given as Additional Data); 2) the other combining these characters with the 82 osteological and external characters of FROST et al. (2001) (data set II: 20 taxa x 244 characters). We also compiled and analyzed a data set III incorporating the 48 taxa surveyed by FROST et al. (2001) and including all 244 characters. Since we were unable to perform muscular dissections of all species analyzed by FROST et al. (2001) (48 taxa), in data set II we coded as missing entries the character states of those taxa we could not examine. Our discussion focuses on the result from the analysis of data set II, since it includes almost all the characters for all the 20 taxa considered.

As a way to keep our data set II analysis as similar as possible to that of FROST et al. (2001), we used not only *Polychrus* sp. and *Anolis* sp. (Daudin, 1802), but also corytophanids, Scleroglossa, Opluridae, and *Leiocephalus* sp. (Gray, 1825) as outgroups in the second data set, exactly as they were used in FROST et al. (2001). For this purpose, for the latter three taxa we used the osteological and external morphology characters provided by FROST et al. (2001). Regarding the corytophanid *Basiliscus vittatus*, we used the characters provided by FROST et al. (2001) plus the new myological data obtained by us. As stated above, our primary focus in this study was the leiosaurids, but we did include one of the *Anolis* species studied by FROST et al. (2001): *Anolis carolinensis*. Furthermore, we dissected other *Anolis* specimens, belonging to fourteen species, in order to evaluate anatomical variability among them, although we purposely did not include all of them in our data set, so as to keep our data set similar to that of FROST et al. (2001). Thus, we included 4 *Anolis* species, so our data set consisted of only 20 taxa. Our dissections revealed constancy of myological structures in *Anolis* specimens and we are thus confident that the characters used are an appropriate representation of the variation in the genus (see below). Of the 162 myological characters included in our phylogenetic analysis, 90 are informative (i.e. they provide evidence to enable inferences about relationships between the terminal taxa used) and 72 are uninformative because they are invariant. Although the latter do not provide direct information about relationships between terminal taxa, they are useful in documenting the distinctive attributes within these taxa. Thus, by including uninformative characters in a matrix, relevant anatomic information is being considered and documented (see e.g. DIOGO, 2004a). Muscular names for the hindlimb characters follow RUSSELL (1988; 1993).

All three data sets were analyzed using the TNT program (Tree Analysis using New Technology; GOLOBOFF et al., 2003a), with maximum parsimony as the optimality criterion. All three data set analyses were conducted by generating 500 Wagner trees and then submitting them to the tree bisection-reconnection branch-swapping method (TBR), as well as Nixon's ratchet method (NIXON, 1999). With this last method it is less likely to become trapped in islands of suboptimal trees. We used jackknifing and bootstrapping to estimate the support for the branches. Standard bootstrapping is influenced by uninformative characters (and by characters irrelevant to monophyly of a given group) (HOVENKAMP, 2004). Since our data set has many uninformative characters, we rather based our discussion on the jackknife support values. Bootstrap support values are given on Fig. 2, and jackknife support values in Appendix 3.

In order to evaluate the topological congruence between our morphological data set and the results for the molecular data of FROST et al. (2001), we calculated a semi-strict supertree (GOLOBOFF & POL, 2002) combining tree topologies with different taxon sets. We decided to use this methodology since we were unable to obtain the original molecular data set of FROST et al. (2001). We compared and contrasted all morphological data, ours and that of FROST et al. (2001), with the molecular data of FROST et al. (2001) in order to avoid analyzing their morphological data twice. Thus, tree topologies resulting from the molecular analysis of FROST et al. (2001) (21 taxa) and our data set II (20 taxa) were combined. A semi-strict supertree displays all the groups that are implied by at least some combination of input trees and contradicted by none (GOLOBOFF & POL, 2002). It should be noted that this amounts to producing a consensus tree, rather than an actual phylogenetic hypothesis.

RESULTS AND DISCUSSION

The analysis of data set I (20 taxa x 162 char.) resulted in a single most parsimonious tree with 326 steps (Fig. 1), while the analysis of data set II (20 taxa x 244 char.) resulted in a single most parsimonious tree with 493 steps (Fig. 2). In both trees, three nodes are well supported (Appendix 3), with jackknife and bootstrap support values of 100% (*Anolis*, *Enyalius*, and *Diplolaemus* nodes), which suggest that the monophyly of these taxa is a sound hypothesis. Most of the nodes received similar values with both support measures, except nodes 29 and 32 that received no support with bootstrap. In general, deeper nodes have lower support values (e.g. leiosaurines have a jackknife value of 35%), although there are some exceptions, such as the Leiosauridae, which is supported by a jackknife value of 63% (Appendix 3). The analysis of the complete morphological data set of FROST et al. (2001) plus our data (data set III: 48 taxa x 244 char.), resulted in five most parsimonious trees, with 712 steps. The strict consensus of these five trees is shown in Fig. 3. By combining the tree topologies arising from the analysis of the molecular data of FROST et al. (2001) and the tree from our data set II, we obtained a semi-strict supertree (Fig. 4).

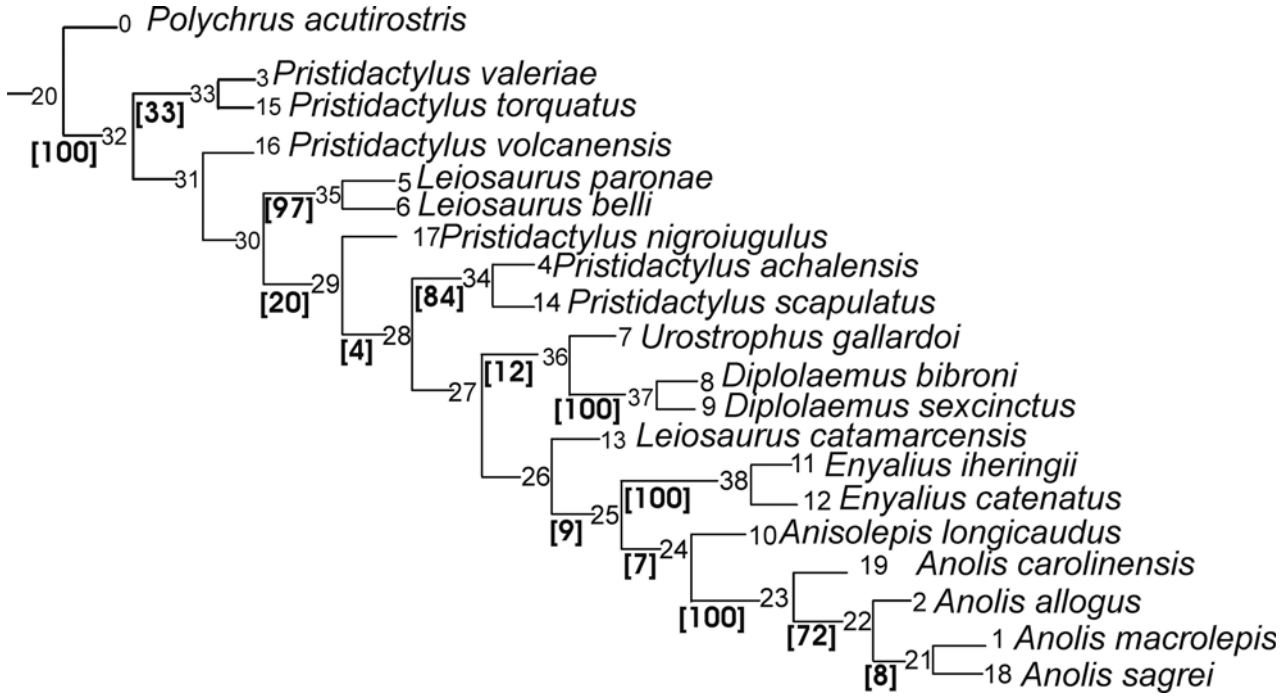


Fig. 1. – Tree generated from analyzing data set I (20 taxa x 162 all myological characters). Node numbers and bootstrap support values (in square brackets) are shown. Nodes without indicated values have no support.

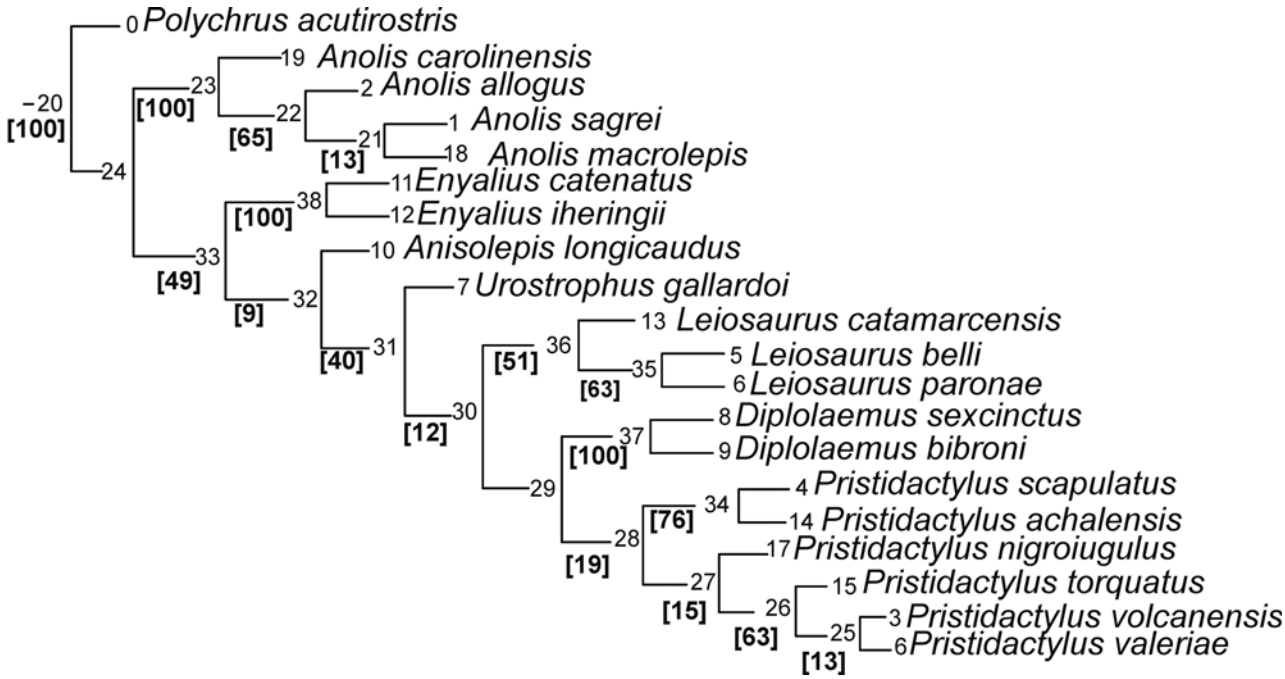


Fig. 2. – Tree generated from analyzing data set II: myological data plus morphological characters of FROST et al., 2001 (20 taxa x 244 characters). Node numbers and bootstrap support values (in square brackets) are shown. Nodes without indicated values have no support.

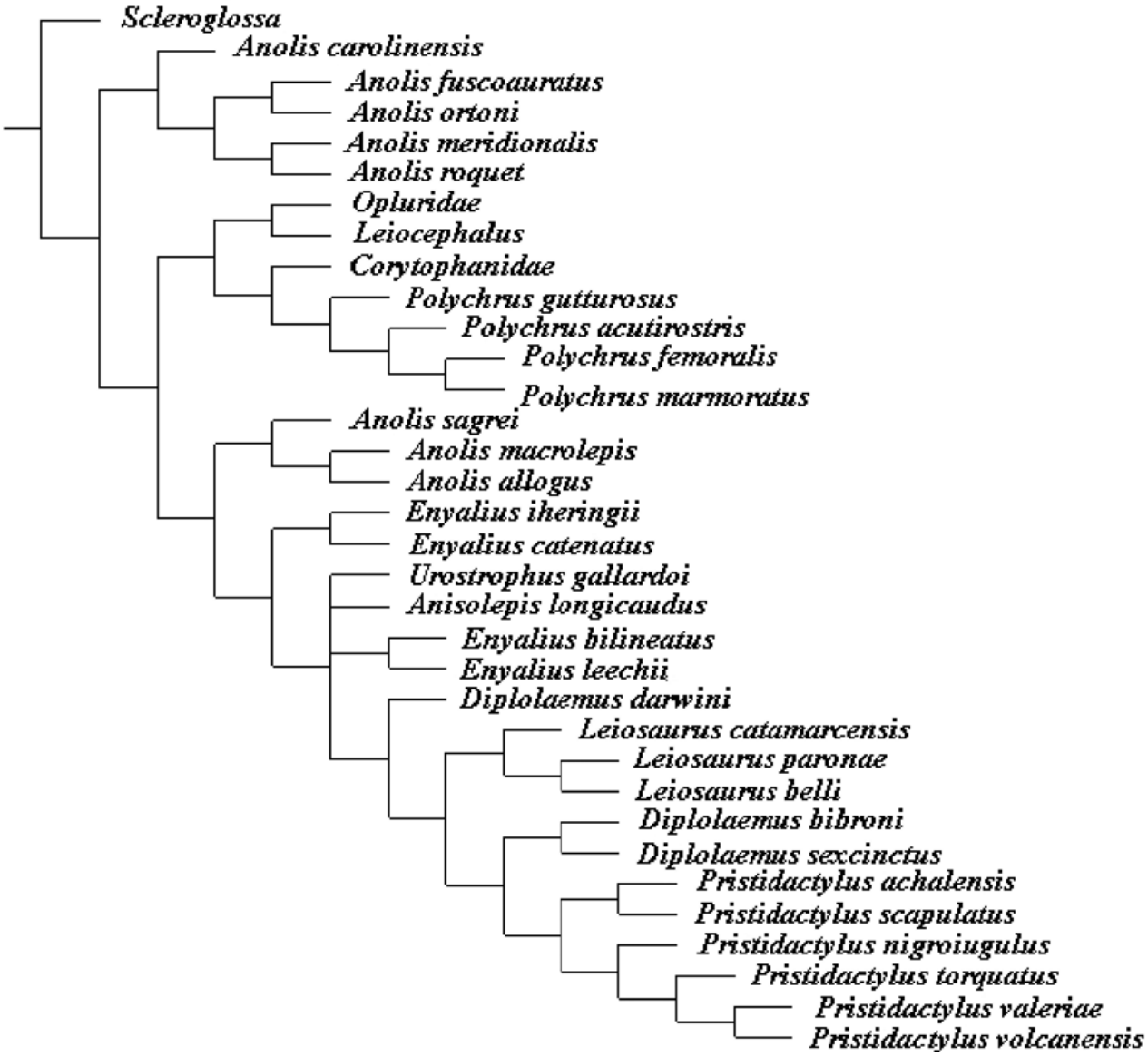


Fig. 3. – Strict consensus of 5 equally most-parsimonious trees generated from analyzing data set III using FROST et al.'s, (2001) morphological characters plus our myological characters (48 taxa x 244 characters).

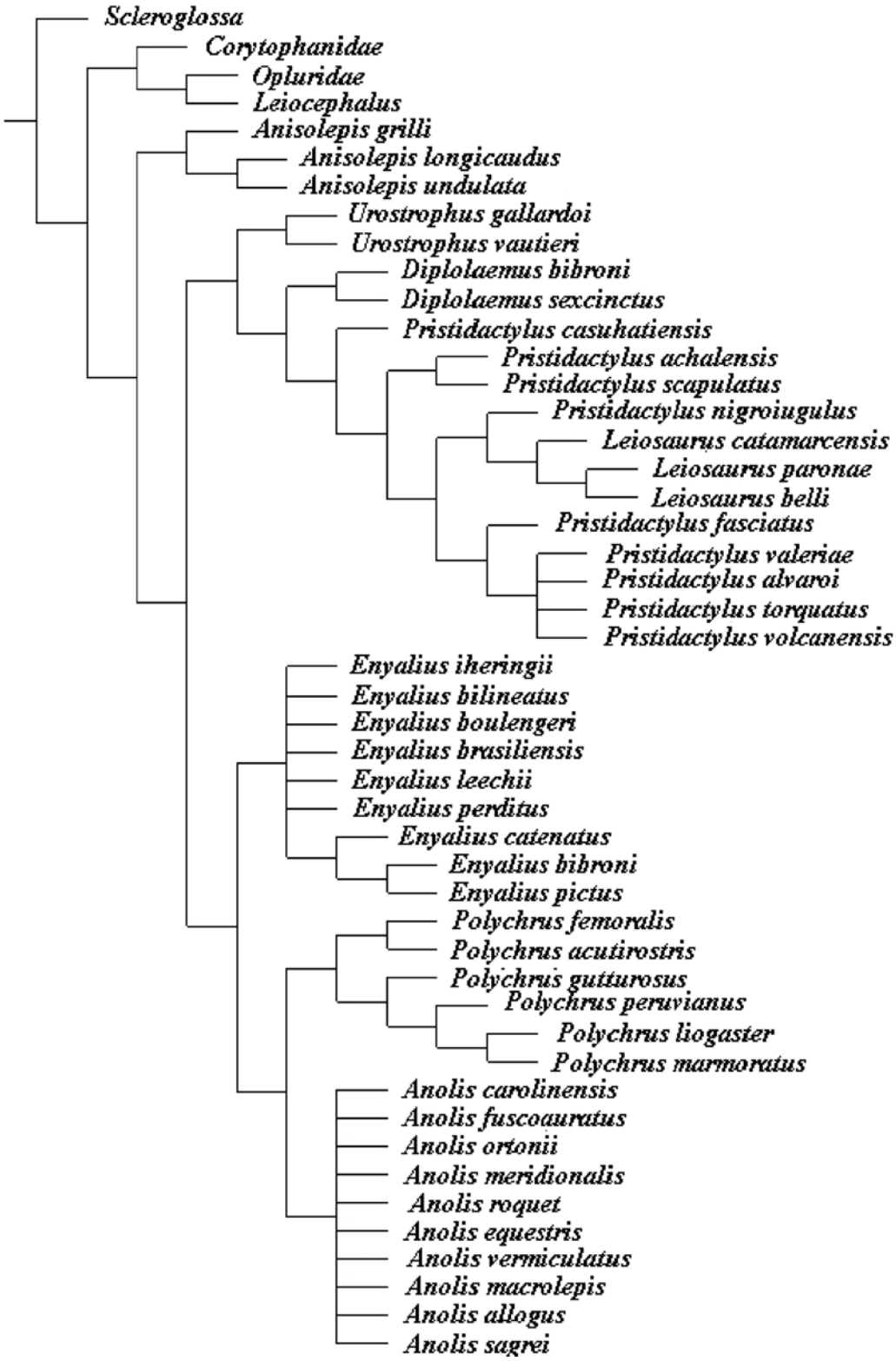


Fig. 4. – Semi-strict supertree, that results from combining tree topologies obtained from the molecular data set of FROST et al. 2001 analysis and from our data set II. Only two higher taxa are recovered as monophyletic groups: Polychrotidae and Leiosaurinae.

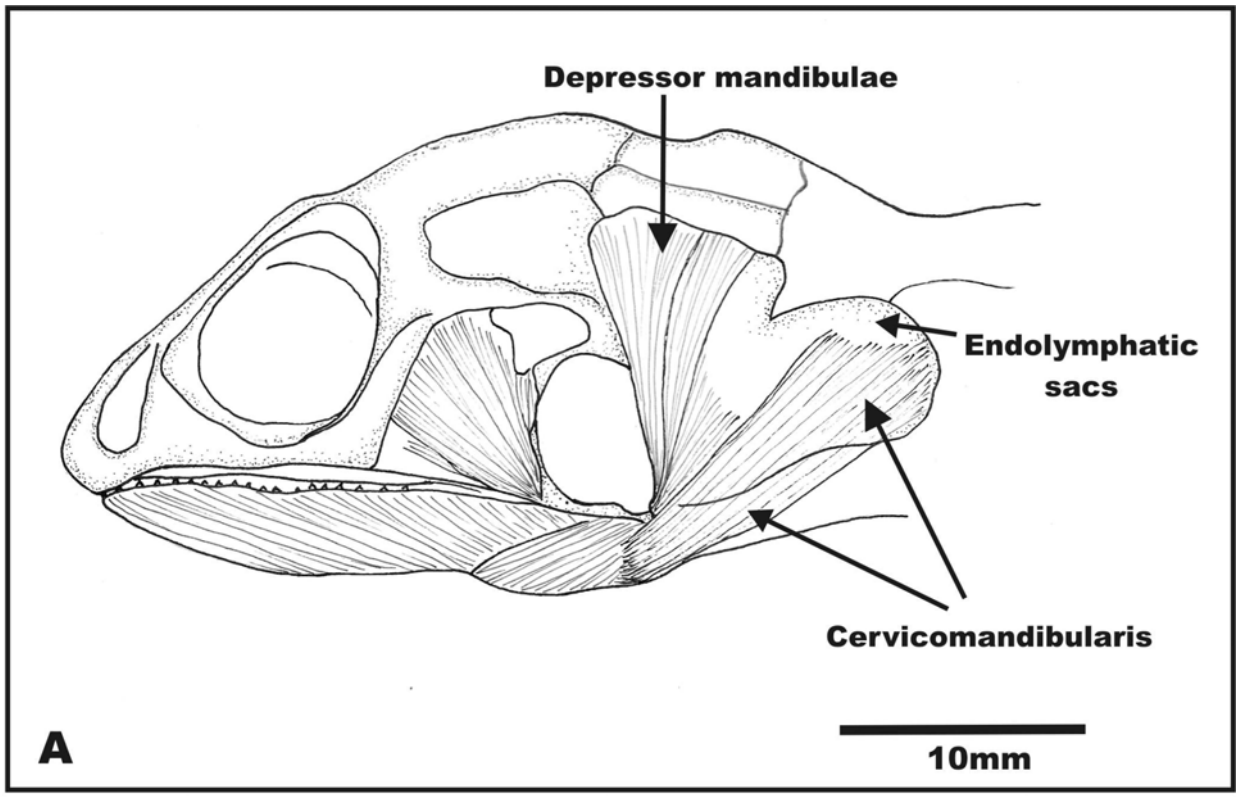
The results of our analysis of data set II indicate that, within the Leiosauridae, *Leiosaurus* genus is characterized by six unambiguous myological synapomorphies (Appendix 3), five of them associated with gular structures. Most of the derived characters of *L. catamarcensis* (Koslowsky, 1898) are highly homoplastic, i.e. they were independently acquired by other taxa (Appendix 3). Only eight of these derived characters are actually exclusively present in this species. Therefore, *L. catamarcensis* provides an interesting case of mosaic evolution (see e.g. GOULD, 2002; DIOGO, 2004a), combining peculiar autapomorphies with features that are also homoplasically found in other lizard species such as *Enyalius iheringi* (Boulenger, 1885). This is particularly interesting because these species apparently do not share similar locomotor modes, or microhabitat use, or any other ecological or ethological trait that could, in theory, be interpreted as constraining their morphology towards a homoplastic configuration. *Leiosaurus* sp. is a ground-dwelling lizard that inhabits mainly arid and semidesertic regions of Argentina (CEI, 1973). Lizards of the genus *Enyalius* are restricted to forested areas along the Atlantic Rainforest of eastern Brazil and the Brazilian Amazon forest, and are usually found using tree trunks, shrubs, fallen logs or leaves as perches (VAN SLUYS et al., 2004).

The enyaliine leiosaurid *Urostrophus* specimens analysed have a divided m. depressor mandibulae and a hypertrophied m. cervicomandibularis (Fig. 5a), almost twice the width of this muscle in e.g. *Anisolepis* (Boulenger, 1885) specimens (Fig. 5b). In general, the cranial musculature of *Urostrophus* specimens has a somewhat simplified configuration, many muscles being absent, e.g. the m. adductor posterior and m. mandibulohyoideus III. According to our phylogenetic analysis, in the case of the enyaliine taxon *Anisolepis,* 12 out of 30 character states are seemingly homoplastic parallelisms that are also found in closely related taxa (Appendix 3). Three of the six unique autapomorphies present in this taxon (Appendix 3) are modifications of upper limb muscles. One of these unique features is the absence of m. pronator teres, which is noteworthy considering that this muscle usually promotes the external rotation of the forearm. Another unique feature of *Anisolepis* genus concerns the m. pronator profundus, which occupies only half of the distal space between the radius and ulna, and not all this distal space, as seen in the other lizards analyzed. These peculiarities related to both the m. pronator teres and the m. pronator profundus make *Anisolepis* genus an interesting case study for conducting functional and ecomorphological studies on the relations between the seemingly peculiar limb rotation movements displayed by this taxon and the type of environment in which it lives.

The clade composed by *Anolis* species is defined in our analysis by 11 unambiguous myological synapomorphies. Three of these synapomorphies are related to structures associated with the dewlap support (Appendix 3). In general, the cranial ventral musculature is modified in *Anolis* specimens, probably in association with the big size of the second ceratobranchials (Fig. 6). The ventral gular skin is adhered to this portion of the hyoid. In some specimens of *Anolis gundlachi* (Peters, 1876) examined by us (e.g. RT 144478), these hyoid structures reach the pelvic girdle in a resting position; in others (e.g. RT 14487, juvenile specimen) they reach the shoulder girdle. Dewlap size is known to vary ontogenetically and between sexes in many species of *Anolis* (FITCH & HILLIS, 1984; NICHOLSON et al., 2007); the difference in the length of the second ceratobranchials was already noted by FROST et al. (2001) in relation to the presence of dewlap (their char. 22). In *Anolis* specimens, the second ceratobranchials are partially covered ventrally by the m. constrictor colli (Fig. 6), which forms a continuous layer with the m. intermandibularis anterior and m. intermandibularis posterior. It is difficult to differentiate the m. constrictor colli from the m. intermandibularis posterior near their insertion on the mid-ventral fascia (Fig. 6). This ventral fascia formed by the two muscles reaches the most distal part of the head, covering the m. pterygomandibularis (Fig. 6).

In *Polychrus* specimens the second ceratobranchials are covered by the m. intermandibularis posterior, which is loosely attached to the skin (not shown). One main difference between the polychrotids and the leiosaurid type genus *Leiosaurus* concerns the position of the second ceratobranchial. In *Anolis* and *Polychrus* specimens, this bone is very superficial and does not have muscular fibers attached to it, while in *Leiosaurus* specimens it is deeply embedded in the muscular fibers of different hyobranchial muscles.

In the specimens of the leiosaurid *Anisolepis* the flexor plate with its palmar sesamoid is smaller than it is in taxa belonging to node 30 (e.g. *Leiosaurus* sp., Fig. 7a, b). The reduction in size of the palmar sesamoid is correlated with the independence of the flexor tendons, as is shown in *Anolis* specimens (Fig. 7c), which prevents the formation of a unique flexor plate. The presence of a palmar sesamoid embedded in the tendinous tissue probably prevents the complete closure of the manus because of its rigidity; that probably precludes, in turn, palmar flexion (pattern L of MORO & ABDALA, 2004).



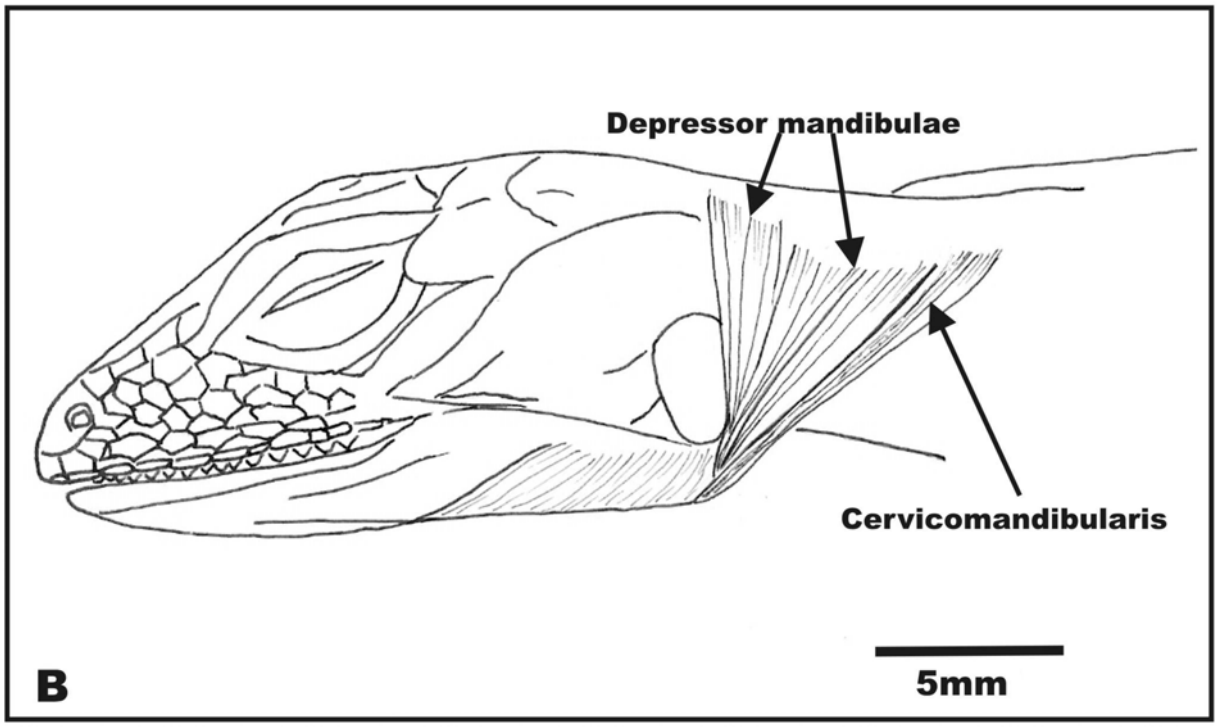


Fig. 5. – A. Lateral view of the cephalic musculature of *Urostrophus gallardoi*; note the hypertrophied m. cervicomandibularis covering the endolymphatic sacs. This is almost twice the width of this muscle in *Anisolepis longicaudus*. B. Lateral view of the cephalic musculature of *Anisolepis longicaudus*, with a normal m. cervicomandibularis.

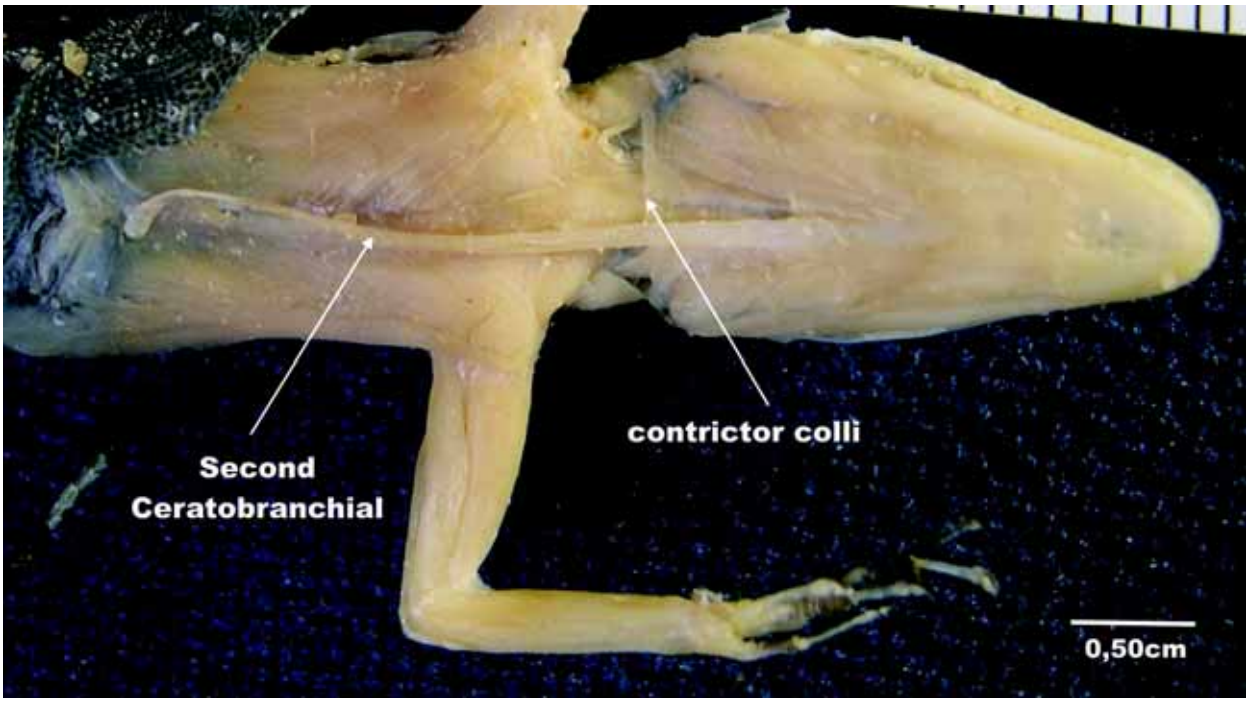


Fig. 6. – Photograph of the ventral view of the anterior region of *Anolis gundlachi*; note the very superficial location of the second ceratobranchials, partially covered by the m. constrictor colli.

Comparison among the different hypothesis.

The comparison of the tree topologies resulting from the combination of FROST et al. (2001) analysis and our own myological data reveals some interesting points that will be discussed below.

DATA SET I. The analysis of this data set, including only myological characters, results in a tree with most of the genera often recognized in the literature appearing as monophyletic clusters, except *Leiosaurus* and *Pristidactylus,* with most of the analyzed species belonging to this genera. This is particularly interesting, because some previous studies on a wide range of vertebrate taxa including lizards (e.g. MORO & ABDALA, 1998; ABDALA & MORO 1996; 2003; 2006) as well as bony fish, birds, and primates (see e.g. DIOGO, 2004b, for a recent review of this subject) have indicated that the analysis of muscular characters was more likely to reveal synapomorphies for higher taxa such as families and orders than for lower taxa such as species or genera. In fact, in the present study, the exclusive analysis of myological characters (data set I) did not recover any of the higher-level taxa (above the genus level) that are often recognized in the literature. RUSSELL (1988) stressed that myological features should be approached and used with caution, especially at higher taxonomic levels because of homoplasy. All these contrasting results support the contention that myological data should be used with caution, indicating that the best option in morphological cladistic analysis thus continues to be trying to complement the evidence provided by hard tissues and that provided by soft structures, as well by other types of data (e.g. external features), i.e., to analyze all the anatomical data available.

DATA SET II. In fact, contrary to the analysis of our data set I, the analysis of data set II combining our own 162 myological characters with the 82 osteological characters of FROST et al. (2001) did recovered the monophyly of the family Leiosauridae, as well as of all its genera (Fig. 2). The family Polychrotidae and the subfamily Enyaliinae are recovered as paraphyletic taxa. In the consensus tree obtained from the morphological data set by FROST et al. (2001; see their Fig. 2), all enyaliine genera were grouped in a monophyletic unit, but their relationships appeared as unresolved. This group (Enyaliinae or Anisolepae sensu SCHULTE et al., 2003) should therefore be accepted with caution.

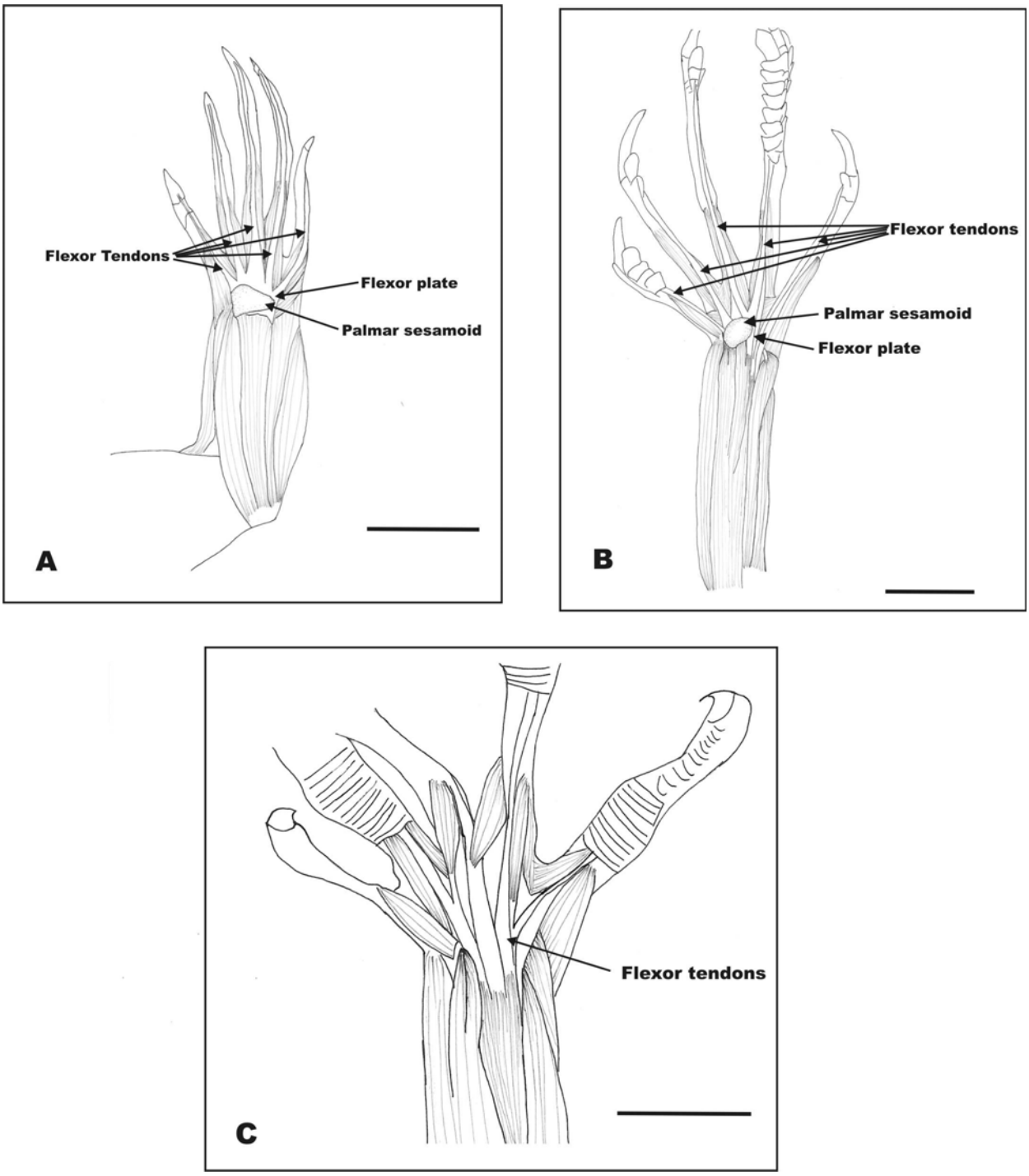


Fig. 7. – A. Ventral view of the manus of *Anisolepis longicaudus*; the flexor plate with its palmar sesamoid is smaller than it is in *Leiosaurus*. B. Ventral view of the manus of *Leiosaurus catamarcensis*; note the big palmar sesamoid, which probably prevents the flexion of the palm of the hand. C. Ventral view of the manus of *Anolis gundlachi.* There is no developed flexor plate.

In the analysis of data set II, *Leiosaurus* genus appears as monophyletic, with a 51% bootstrap support value. FROST et al.'s (2001) study for all data did recover this genus as a polytomy; the inclusion of myological characters on data set II has thus contributed to solve that polytomy (Appendix 3) with the synapomorphies commented above. Regarding the Pristidactylinae, although we recovered it as monophyletic, this clade has essentially no support (bootstrap support 12%) in our analysis of data set II.

DATA SET III. Although the overall analysis of data set III recovers the family Polychrotidae as monophyletic, its unexpected phylogenetic position on the tree (Fig. 3, data set III) suggests that it is crucial to assemble more evi-

dence before the Polychrotidae - Leiosauridae relationships can be considered settled. When FROST et al. (2001) added molecular characters to their morphological data set, they obtained a more resolved consensus tree (see their Fig. 4). When we analyze the data set incorporating the 48 taxa surveyed by FROST et al. (2001) and including our own 162 myological characters and the 82 osteological characters of FROST et al. (2001) (data set III), monophyly of the Polychrotidae is again obtained, and the Leiosauridae and Enyaliinae appear as paraphyletic groups (Fig. 3). Only *Leiosaurus* and *Pristidactylus* appear as monophyletic within the Leiosaridae.

SUPERTREE. Only two higher taxa are recovered as monophyletic groups: Polychrotidae and Leiosaurinae. *Leiosarus* genus is once again recovered as monophyletic. Leiosauridae, Enyaliinae and *Pristidactylus* genus appear as paraphyletic groups (Fig. 4).

GENERAL COMMENTS. The Enyaliinae appear as a paraphyletic group in all our analyses. The other taxa analyzed appear as paraphyletic in some analyses and monophyletic in others. Interestingly, in both FROST et al.'s (2001) and SCHULTE et al.'s (2003) studies and in our analyses of data sets II and III, the Leiosauridae appears as a monophyletic group. FROST et al. (2001) and SCHULTE et al. (2003) recovered the monophyly of Enyaliinae and Leiosaurinae, although SCHULTE et al. (2003) use different names for these clades. We recovered only Leiosaurinae as monophyletic in the analysis of data set II, III, and in the supertree. With respect to the lower taxa, *Leiosaurus* genus is recovered as monophyletic by FROST et al. (2001), using only morphological characters, by SCHULTE et al. (2003) and in all our analyses, except that based on our data set I. *Diplolaemus* genus is recovered as monophyletic by SCHULTE et al. (2003) and in all our analyses. *Pristidactylus* genus is recovered as a clade by FROST et al. (2001), and by our analyses of data sets II and III, while *Enyalius* genus appears as monophyletic in FROST et al. (2001) study, and in our analyses of data sets I, II and III. Lastly, *Anisolepis* and *Urostrophus* genera are monophyletic in FROST et al. (2001) study, and in our supertree.

In summary, of the taxa discussed in the present work, there are three taxa that consistently appear as monophyletic groups in at least some of the analyses made by both FROST et al. (2001), by SCHULTE et al. (2003), and by us: the Leiosauridae, Leisaurinae, *Diplolaemus*, and the type genus of this family, *Leiosaurus*. Therefore, it can be said that in view of the data available, the monophyly of these four taxa is particularly well supported.

ACKNOWLEDGEMENTS

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Appendix 1: Specimens analyzed

Acronyms: FBC, Félix Benjamin Cruz in Proyecto Tupinambis, Argentina; FML, Fundación Miguel Lillo (Argentina); MACN, Museo Argentino de Ciencias Naturales, Buenos Aires (Argentina); MNHN, Museum National d' Histoire Naturelle, Paris (France); MZUSP: Museu de Zoología Universidade de São Paulo (Brazil); NMW, Naturhistoriches Museum, Wien (Austria); PT, Proyecto Tupinambis, Tucumán (Argentina); RT, private colection of Richard Thomas (Puerto Rico); SDSU, San Diego State University (USA); UNNEC: Universidad Nacional del Nordeste (Argentina); MMHN, Museo Municipal de Historia Natural, Mendoza, (Argentina).

Leiosaurinae

Diplolaemus sp. (3 specimens): FBC 53-55: 2/3/99. Río Negro, 67. 40º 26.955 S and 68º 22.613 W; (1 specimen) PT 4832: 21/2/99. Same data.

Diplolaemus bibroni (Bell, 1843) (1 specimen): MACN 35850 SN 43: 10/80. Santa Cruz, Argentina; (1 specimen) SN 29: 4/11/91. Forma alto patagónica. Somuncurá, Río Negro, Argentina.

Diplolaemus sexcinctus (Cei, Scolaro & Videla, 2003) (1 specimen): FML 16988. Puesto Rojas. Argentina.

Leiosaurus paronae (Peracca, 1897) (1 specimen): MACN 4386, no data.

Leiosaurus belli (Duméril & Bibron, 1837) (1 specimen): NMW 12976, no data; (2 specimens) PT 3998-3999: 4-9/12/98. Río Negro, Argentina; (1 specimen) PT 4782: 2/2/99. 2 km Río Negro, Argentina; MMHN 403, 406-408. Mendoza, Argentina (4 specimens).

Leiosaurus catamarcensis (2 specimens): FBC 104-105: Santa María, Argentina. 26º 59.358 S and 66º 16.484 W; (1 specimen): FBC 145: 16/3/99. La Rioja, Argentina; (1 specimen): PT 3715: 1-2/11/98. La Rioja.

Pristidactylus volcanensis (Lamborot & Díaz, 1987) (2 specimens): MNHN: no number. El Volcán (Chile).

Pristidactylus valeriae (Veloso & Navarro, 1988) (1 specimen): FML no data.

Pristidactylus torquatus (Philippi, 1861) (2 specimens): NMW 18198, 18199, no data.

Pristidactylus achalensis (Gallardo, 1964) (1 specimen): MACN 32779: 1/83. Córdoba, Argentina. Stranech, Carrizo col.

Pristidactylus scapulatus (Burmeister, 1861) (1 specimen): MACN 35370: 3/93. San Juan, Argentina.

Pristidactylus nigroiugulus (Cei, Scolaro & Videla, 2001) (1 specimen): FML s/n:7/3/03. Chubut. Scolaro, col.

Enyaliinae

Enyalius iheringii (1 specimen): MZUSP 74901: 19/11/91. Boracéia, FAG. Mello, Vanzo det.

Enyalius catenatus pictus (Jackson, 1978) (1 specimen): 16-28/II/86. Reserva Biológica Pau Brasil, Ba. M. Rodriguez 86.6024. M. Rodriguez det.

Anisolepis longicauda (Boulenger, 1891) (1 specimen): UNNEC: no data.

Urostrophus gallardoi (Etheridge & Williams, 1991) (3 specimens): FBC 127-129: Córdoba; (1 specimen) FBC 0036: 2/3/99. Córdoba, Argentina.

Polychrotidae

Anolis olsseni (1 specimen): SDSU 2164: 1953. Port au Prince, Haiti. R. Etheridge col.

Anolis sagrei (Duméril & Bibron, 1837) (1 specimen): SDSU 2175: 1953. Key West, Florida, USA. R. Etheridge col.

Anolis lineatopus (Gray, 1840) (1 specimen): SDSU 2157: 1953.Kingston, Jamaica. R. Etheridge col.

Anolis cristatellus (Duméril & Bibron, 1837) (1 specimen): SDSU 2145: 1953. San Juan, Puerto Rico. R. Etheridge col.

Anolis coelestinus (Cope, 1863) (1 specimen): SDSU 2148: 1953. Port au Prince, Haiti. R. Etheridge col.

Anolis allogus (Barbour & Ramsden, 1919) (1 specimen): SDSU 2136: US Bay Naval Base, Guantanamo, Cuba. R. Etheridge col.

Anolis carolinensis (2 specimens): FML no data.

Anolis macrolepis (Boulanger, 1911) (1 specimen): SDSU 2183: 24/7/68. Cano Decoraro, Chocó, Colombia. E.E. Williams col.

Anolis notopholis (Boulanger, 1896) (1 specimen): SDSU 2188: Cano Decoraro, Chocó, Colombia. E.E. Williams col.

Anolis gundlachi (9 specimens): RT 14476-14484: Bosque Carite, Puerto Rico.

Anolis cuvieri (Merrem, 1820) (1 specimen): RT 59694: Puerto Rico.

Anolis sp. (5 specimens): RT 14485-88, 14491: Reserva El Verde, Puerto Rico.

Anolis krugi (Peters, 1876) (2 specimens): RT 14489-90: Bosque Carite, Puerto Rico.

Anolis stratulus (Cope, 1861) (1 specimen): RT 14492: Reserva El Verde, Puerto Rico.

Polychrus acutirostris (Spix, 1825) (10 specimens): FML 00140: Jujuy, Argentina. MZUSP 08605, 08606, 08610, 08611: Pesqueira, Pe., Brazil. MZUSP 48166: Fazenda Babente, 13 Km E Exu, Pe., Brazil. MZUSP 48151, 48154, 48156: Exu, Pe., Brazil.

Polychrus sp. (1 specimen): MACN 7292: Paraguay.

Corytophanidae

Basiliscus vittatus (Wiegmann, 1828) (1 specimen): SDSU 02097 no data

Appendix 2: Myological Data set, taxa and character codes (Polymorphism symbols: A=0 and 1; B=0 and 2; C=2 and 3; D=1 and 2; E=1 and 4; F=0 and 3; G=0 and 4; H=1 and 3; J=3 and 4)

	1		2		3		4		5		6		7			
	01234	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**
Anolis allogus	22002	00---	10102	10-12	1-220	-1102	02101	010-1	11011	0-000	11111	02001	0-1--	-1001	11022	2----
Anolis macrolepis	02000	00---	10102	10-12	1-220	-1102	02101	010-1	11011	0-000	11111	02001	0-1--	-1001	11022	2----
Anolis sagrei	02002	00---	10102	10-12	1-220	-1102	02101	010-1	11011	0-000	11111	02001	0-1--	-1001	11022	22---
A. carolinensis	22113	02---	00102	10-12	1-200	-1102	00101	01110	21011	0-000	01101	02001	0-1--	-1001	11022	22---
U. gallardoi	12100	02---	10112	10-12	0-200	-1101	020--	-10-1	22001	1-002	01110	02001	112-0	02001	11003	21110
D. bibroni	12100	02---	10112	10-12	0-200	-1101	02101	010-1	10001	0-000	11110	02020	1---1	01001	11003	11111
D. sexcinctus	12100	02---	10112	10-12	0-200	-1101	02101	010-1	10001	0-000	11110	02020	1---1	01001	11003	11111
A. longicaudus	22A03	1-01-	00102	11-12	12-00	-1102	0211-	010-1	01011	2-002	01111	02000	1---0	01001	G1003	21100
E. iheringii	22101	----1	00112	11-12	1-200	-1101	020--	-10-1	12001	0-000	11111	02000	1---0	01001	01002	11111
E. catenatus	22101	----1	00112	11-12	1-200	-1101	020--	-10-1	12001	0-000	11111	02000	1---0	01001	11002	11111
P. scapulatus	12002	02---	00112	11-12	0-200	-1100	02101	01111	10001	0-002	01011	02000	1---1	01001	00003	11111
P. achalensis	12002	02---	00112	10-12	0-200	-1100	02101	01111	00001	0-002	01011	02000	1---1	01001	00003	11111
P. nigroiugulus	02100	02---	10102	11-11	2-201	01101	02101	01110	20000	0-002	01011	10000	111--	01001	00003	21111
P. valeriae	02100	02---	10102	10-12	1-001	01001	02101	01100	20010	0-002	01010	10101	111--	00--1	11002	1110-
P. torquatus	02100	02---	10102	10-12	1-301	01001	02101	01110	20010	0-002	01010	10101	111--	01101	21002	1110-
P. volcanensis	02100	1-0A-	10102	11112	1-B01	01A01	02101	011AA	20010	0-002	01010	10101	111--	01A0D	11002	2110-
L. catamarcensis	12100	02---	00110	11-12	0-200	-0101	02011	010-1	52000	1-000	01011	10100	----1	01002	01002	21111
L. paronae	01001	1---2	10102	11-12	0-101	01001	02101	01100	D2010	1-010	01010	10100	----1	0---1	11010	D111-
L. belli	02100	1-0A-	00102	11-12	0-F01	0100D	02101	01100	D2010	1-010	01010	10100	----1	01001	11010	D111-
Polychrus	02100	1---1	00112	11-12	11-01	01100	02100	01100	20010	0-002	01011	10101	110--	01001	11001	11100

	8		9		10		11		12		13		14		15		16
	01234	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01234**	**56789**	**01**
Anolis allogus	--100	101-1	00011	20010	00121	10001	-0200	10021	00010	00101	01010	10000	11001	00000	00020	01011	11
Anolis macrolepis	--100	101-1	00011	20010	00121	10001	-0000	11021	00010	00101	01010	10000	11001	00000	00020	01011	11
Anolis sagrei	--100	101-1	00011	20010	00121	10001	-0000	11021	00010	00101	00000	00100	11001	00000	00020	01011	11
A. carolinensis	--100	101-1	00011	20010	00121	10001	-0000	11021	00010	00101	00000	00100	11001	00000	00020	01011	11
U. gallardoi	-0000	000-0	11100	00A10	00132	30041	-0001	001F0	00000	0A111	00000	10000	11011	00000	10000	10010	00
D. bibroni	-1001	001-0	02000	10010	0AADD	HA1J1	00F00	D0A01	000AA	B0A10	-0000	10000	11011	00001	10020	110-0	00
D. sexcinctus	-1001	001-0	02000	02000	0AADD	HA1J1	00F00	D0A01	000AA	B0A10	-0000	10000	11011	00001	10020	110-0	0
A. longicaudus	01100	002-1	A1000	20010	00121	30040	00000	10131	00010	100-0	-0000	10000	11101	11000	11000	01100	02
E. iheringii	-1000	100-1	00000	22110	01101	10041	00001	20101	10011	01121	10000	--000	11001	00001	10120	02022	02
E. catenatus	-1000	100-1	00000	22110	00101	10141	00001	00120	10011	01121	10000	10000	11001	00001	10120	02022	02
P. scapulatus	-1100	000-1	11100	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	--
P. valeriae	-1100	000-1	11100	20010	00122	30101	00100	00110	00010	10--1	00000	10000	11211	00001	10020	01010	00
P. achalensis	-1110	000-1	1-000	10010	00102	30101	00001	11001	00000	10111	00000	10000	11-11	00001	10020	01010	00
P. nigroiugulus	-1110	000-0	12000	-0010	001-2	30101	00-0-	1-0-1	000-0	10111	00000	10000	11-11	00001	10020	01010	00
P. torquatus	-1110	000-0	11000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	--
P. volcanensis	-1110	000-0	14000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	--
L. catamarcensis	-1110	004-2	14100	20011	11002	30111	10201	20030	00010	00101	00000	01000	11111	20101	10120	00110	00
L. paronae	-1010	000-0	14000	00010	00102	30001	00000	10001	00000	10--0	-000-	0-100	11-11	00001	10000	00000	00
L. belli	-1110	000-2	1-000	10010	00002	30000	00000	10001	00000	10111	00000	1-200	11-10	00--1	----0	00000	00
Polychrus	-1100	001-0	0-000	11010	00A01	00000	00100	10101	00011	00100	-1010	10200	10001	00000	21-10	12120	00

Appendix 3: List of synapomorphies of the main nodes with their jackknife support values (nodes as Fig. 2; Ja=Jackknifing; No=Node)

No	Ja	Synapomorphies
21	42	• Adductor aponeurosis (character 0): very nacreous → not pigmented
22	82	• Temporal fossa aponeurosis (character 2): pigmented → not pigmented • Levator anguli oris insertion(character 10): with aponeurosis → without aponeurosis • Temporal artery (character 23): visible → almost invisible • Levator pterygoidei length (character 50): long → short
23	100	• Tendinous system of the adductor mandibulae complex present (character 13): very developed → developed • Adductor superficialis externus origin (character 16): extends on postorbital, squamosal and quadrate → extends on jugal, postorbital, squamosal, and quadrate • Adductor mandibulae externus profundus origin (character 29): quadrate and prootic → quadrate, prootic, and parietal • Depressor mandibulae anterior origin (character 60): does not include supraoccipital → includes supraoccipital • Mandibulohyoideus (character 73): mid region of the dentary → posterior region of the dentary • Omohyoideus (character 93): unique → divided • Modified mandibulohyoideus II (character 94): absent → present • Relative size of the extensores digiti brevis/dorsometacarpalis muscles (character 117): dorsometacarpalis = extensores digiti brevis → dorsometacarpalis bigger than extensores digiti brevis • Larger muscle in dorsal view (character 118): extensor digitorum longus → extensor carpi radialis • Tibialis anticus origin (character 159): all fibula length → half distal fibula length • Tibialis anticus (character 160): unique → divided • Digital pads (character 200): absent → present • Sexual size dimorphism (character 207): females larger than males → males larger than females • Coronoid lateral process (character 221): absent or short → large • Splenial posterior extent (character 223): terminates posteriorly anterior to anterior edge of mandibular fossa → terminates posterior to anterior edge of mandibular fossa • Angular (character 224): moderate to large → absent or reduced to splint • Caudal autotomy fracture planes (character 241): absent → present
25	33	• Scale organ of dorsum (character 204): spinules present → without spinules • Dermal roof bone rugosities (character 215): strong rugosities that correspond to scale outlines extend over parietal and frontal and adjacent dermal skull bones → absent or weak, although indistinct rugosities may be present
26	71	• Adductor mandibulae externus medialis insertion (character 27): coronoid and bodenaponeurosis → just bodenaponeurosis • Pterygomandibularis origin (character 43): not divided → divided in two slips with tendon • Intermandibularis anterior profundus – mandibulohyideus II relation (character 57): both muscles attached → not attached • Depressor mandibulae superficialis (character 59): undivided → divided • Mandibulohioydeus I shape (character 74): rectangular → trapezoidal • Mandibulohioydeus II relation (character 78): contralateral muscles joined → contralateral muscles separated
27	27	• Adductor aponeurosis (character 0): pigmented → not pigmented • Tendinous system present (character 13): very developed → developed • Adductor mandibulae externus medialis (character 20): divided → undivided • Position of the temporal artery (character 24): located over two muscles → located over one muscle • Pseudotemporalis superficialis insertion extends over (character 39): both coronoid and bodenaponeurosis → bodenaponeurosis • Pterygomandibularis volume (character 44): flattened → bulky • Intermandibular anterior profundus aponeurosis (character 55): absent → present • Intermandibular anterior profundus shape (character 56): rectangular → irregular • Branchiohyoideus aponeurosis (character 83): absent → present
28	35	• Pseudotemporalis superficialis origin (character 37): includes parietal and postorbital → does not include postorbital • Flexor carpi radialis pattern (character 125): two branches → one branch • Caudal annuli (character 192): irregular → regular • Sexual size dimorphism (character 207): females larger than males → males larger than females • Sexual dichromatism (character 208): absent → present • Black antehumeral bar (character 209): absent → present
29	16	• Pseudotemporalis profundus insertion (character 41): mandibular fossa and coronoid; mandibular fossa, coronoid and bodenaponeurosis → mandibular fossa • Pterygomandibularis aponeurosis (character 45): scarcely pigmented → not pigmented • Mandibulohioydeus I insertion (character 75): ceratobranchial I and epibranchial → ceratobranchial I • Distal subdigital lamellae (character 199): not divided → longitudinally grooved or divided • Caudal autotomy fracture planes (character 241): absent → present, although occasionally showing ventral fusion
30	35	• Depressor mandibulae superficialis origin (character 64): parietal and spinalis capitis → parietal, spinalis capitis, and squamosal • Mandibulohioydeus II insertion including ceratobranchial I (character 79): ceratobranchial I and basihial → ceratobranchial I • Relative of the extensores digiti brevis/dorsometacarpalis muscles (character 117): dorsometacarpalis = extensores digiti brevis → dorsometacarpalis bigger than extensores digiti brevis • Femorotibialis aponeurosis (character 149): fan shaped → rectangular • Supradigital scale shape (character 194): not all supradigitals of third phalanx → all supradigitals at third phalanx as least twice as broad as postdigital of third phalanx • Postdigital scales of third finger (character 196): single lateral row penetrating proximally to penultimate phalanx → triple postdigital row penetrating proximally to penultimate phalanx

No	Ja	Synapomorphies
		• Osseus labyrinth (character 216): high elevation of the osseus labyrinth above the level of the opisthotic → superficial outline of osseus labyrinth distinctly above the level of the opisthotic • Retroarticular fossa (character 227): well developed → reduced • Marginal teeth (character 229): tricuspid → tapered blunt
31	54	• Adductor aponeurosis (character 0): very nacreous → pigmented • Adductor mandibulae externus medialis (character 20): undivided → divided • Limb aponeurosis (character 95): not pigmented → lightly pigmented • Extensor carpi radialis branch number (character 104): 2 → 3 • Palmar patella morphology (character 143): very small → big, flat • Paravertebral scale shape (character 186): polygonal → rounded • Ventral body scales (character 189): unicarinate → smooth • Total caudal vertebrae (character 242-243): 46-64 → 33-44
32	23	• Pterygomandibularis aponeurosis(character 45): not pigmented → scarcely pigmented • Mandibulohioydeus I shape (character 74): trapezoidal → rectangular • Sternohyoideus insertion (character 91): ceratobranchial I and basihyal → ceratobranchial I • Extensor carpi radialis branch development (character 105): supinator+intermedia developed, profundus reduced → all three braches equally developed • Nasal scale-postrostral scale contact (character 166): separated → in contact • Frontal region (character 173): concave → flat or slightly convex • Transverse processes of caudal vertebrae (character 240): do not extend beyond 16 → extend beyond 16
33	63	• Depressor mandibulae superficialis (character 59): divided → undivided • Omohyoideus origin (character 87): clavicular bar and interclavicle → clavicular bar • Extensores digiti brevis pattern (character 108): origin on ulnar; insertion onto proximal extremity of the corresponding metacarpal → origin on ulnar; insertion onto distal end of each metacarpal • Pronator profundus pattern (character 131): divided → undivided • Mental scale (character 164): divided → undivided • Head scale striae (character 174): linear rugosities present → linear rugosities absent • Supraorbital semicircles (character 177): separated by a single row → separated by two or four rows • Splenial anterior extent (character 222): extremely short or absent, not extending anteriorly more than 25% length of tooth row → extend anteriorly more than 25% length of tooth row • Posterior mylohyoid foramen (character 226): on ventral or ventrolateral face of mandible → on medial face of mandible • Sternum anterior extent (character 232): sternum approaches junction of lateral and posterior processes of interclavicle closely → sternum does not approach junction of lateral and posterior processes of interclavicle closely for more than 50% of length of anterior process anterior to the lateral horns of sternum • Posterior coracoid fenestra (character 235): absent → present, marginal, and weak • Sternal ribs (character 236-237): three, with posterior extremity of sternum not elongated to form parallel rods continuous with xiphisternal rods, and bearing third pair of ribs articulating via synovial joints → four
34	87	• Levator anguli oris condition (character 4): wide triangular → narrow rectangular • Adductor mandibulae externus profundus origin (character 29): quadrate and prootic → quadrate • Sternothyroideus (character 92): absent → present
35	71	• Adductor aponeurosis (character 0): pigmented → not pigmented • Levator anguli oris origin (character 5): includes postorbital and jugal → does not include postorbital and jugal • Tendinous system present (character 13): very developed → developed • Position of the temporal artery (character 24): temporal artery located over two muscles → temporal artery located over one muscle • Adductor mandibulae externus medialis insertion (character 27): coronoid and bodenaponeurosis → just bodenaponeurosis • Pseudotemporalis superficialis origin (character 37): includes parietal and postorbital → does not include postorbital • Pseudotemporalis superficialis insertion extends over (character 39): both coronoid and bodenaponeurosis → bodenaponeurosis • Pterygomandibularis origin (character 43): not divided → divided in two slips with tendon • Levator pterygoidei (character 48): well developed → reduced • Mandibulohioydeus I origin (character 73): mid region of the dentary → posterior region of the dentary • Flexor carpi radialis pattern (character 125): two branches → one branch • Flexores brevis superficialis position (character 158): of the digits I, II, and III in the same superficial plane → of digits IV, III, II, and I in the same plane
36	62	• Intermandibularis anterior profundus aponeurosis (character 55): absent → present • Intermandibularis anterior profundus shape (character 56): rectangular → irregular • Intermandibularis anterior profundus-Mandibulohyoideus II relation (character 57): attached → not attached • Branchiohyoideus aponeurosis (character 83): absent → present • Sternohyoideus insertion (character 91): ceratobranchial I → ceratobranchial I, basihyal, and ceratobranchial II • Extensor digitorum longus branch number (character 103): Two or three branches → one branch • Nasal scale-postrostral scale contact (character 166): in contact → separated • Infralabial scale number (character 178-179): 7-7 or fewer → 8-8 to 12-12 • Middorsal scale row (character 185): absent → present but discontinuous • Paravertebral scale surface (character 187): smooth → tuberculate • Subdigital lamellae of toes (character 197): smooth → asymmetrical keels • Dorsal color pattern (character 210): not fleur-de-lis → fleur-de-lis
37	100	• Levator pterygoidei length (character 50): long → short • Intermandibularis posterior insertion (character 58): joined with the contralateral muscle → joined with the contralateral muscle, and fibers joining the dorsal musculature

No	Ja	Synapomorphies
		• Branchiohyoideus origin (character 82): ceratobranchial I and epibranchial I → ceratobranchial I • Ceratohyoideus (character 84): absent → present • Omohyoideus origin (character 87): clavicular bar → clavicular bar and interclavicle • Sternohyoideus aponeurosis (character 90): not pigmented → pigmented • Sternohyoideus insertion (character 91): ceratobranchial I → ceratobranchial I, basihyal, and ceratobranchial II • Epitrochleoanconeus (character 129): present → absent • Extensor digitorum brevis section a origin (character 155): astragalo calcaneo by a tendon → metatarsal V fleshy • Hindlimb length (character 202): medium → short • Dermal roof bone rugosities (character 215): strong → absent or week • Pterygoid teeth (character 228): present → absent • Transverse processes of caudal vertebrae (character 240): extend beyond 16 → do not extend beyond 16
38	100	• Levator anguli oris condition (character 4): wide triangular → narrow triangular • Adductor mandibulae posterior (character 32): present → absent • Levator pterygoidei length (character 50): long → short • Mandibulohioydeus II insertion including ceratobranchial I (character 79): ceratobranchial I and basihial → ceratobranchial I • Branchiohyoideus origin (character 82): ceratobranchial I and epibranchial I → ceratobranchial I • Extensor digitorum longus origin (character 96): with short tendon → with long tendon • Extensor digitorum longus aponeurosis (character 97): present → absent • Extensor carpi radialis insertion (character 114): distal end of the radius → all radius length • Intermetacarpalis I surface (character 120): including almost all area between fingers → except space between digits 5 and 4, and 2 and 1 • Pronator accesorius pattern (character 126): oblique between ulna and radius → parallel between ulna and radius • Epitrochleoanconeus pattern (character 130): origin on humerus → origin not on humerus • Femorotibialis aponeurosis (character 149): fan shaped → rectangular • Popliteus anticus (character 152): absent → present • Tibialis anticus origin (character 159): all tibia length → ¾ distal length of the tibia • Snout orbit relative lengths (character 163): snout length greater than orbit diameter → orbital diameter greater than snout length • Supraocular scales (character 171): not carinate → strongly carinated • Mesoptychial scales (character 180): not conical → conical • Mid-dorsal scale row (character 185): absent → present • Paravertebral scale surface (character 187): unicarinate → tuberculate • Distal subdigital lamellae (character 199): not divided → divided • Hindlimb length (character 202): medium → long • Supratemporal bones (character 218): lateral side of supratemporal process of parietal → more-or-less equally on both sides of the supratemporal process of parietal • Sphenoccipital process (character 220): absent or short → long • Marginal teeth (character 229): tricuspid → tapered blunt

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Major decline of bat abundance and diversity during the last 50 years in southern Belgium.

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ABSTRACT. In order to identify long-term population trends in bats in southern Belgium, we compared results of winter bat banding between 1939 and 1952 to winter bat counts between 1995 and 2008 in 58 hibernacula.

The results show a strong decrease in the populations of *Rhinolophus ferrumequinum, R. hipposideros, Barbastella barbastellus, Myotis dasycneme* and *M. myotis*. In contrast *M. daubentoni* and *M. mystacinus/brandtii/alcathoe* show a numeric increase between these time periods.

The bat diversity within these hibernacula has decreased by half over the last fifty years.

KEY WORDS : Chiroptera, long-term trend, Belgium

INTRODUCTION

Whilst it is generally accepted that bat populations in Western Europe have declined markedly, the detailed figures on each species' decline over several decades are seldom available. An accurate historical record is, however, indispensable to enable the circumscription of any changes that may have come about, and then to allow conservation goals to be set in their wake (FAIRON, 1967; DAAN & GLAS, 1980; RANSOME, 1989; LESIÑSKI et al., 2005).

Although local studies have been conducted on some sites in Wallonia (FAIRON, 1977; HUBART, 1993; FAIRON, 1999; FAIRON, 2001) or some species have undergone more in-depth investigation at the regional scale (FAIRON et al., 1982; FAIRON 1997; FAIRON & BUSCH, 2003), only a single recent publication (LAMOTTE, 2007) has compiled old and recent data for the Walloon Region. Based on a comparison of distributional ranges and species richness on a 10 by 10km grid across the region's entire territory, it is suggested that most of the species' ranges have shrunk. Yet this geographical approach does not account for numerical differences in sizes of populations (JOSEPH & POSSINGHAM, 2008).

The aim of this paper is to document changes that have occurred in bat populations hibernating in fifty-eight underground roosts in the Walloon Region by comparing banding data from the period 1939-1952 with data obtained recently from censuses conducted between 1995 and 2008.

MATERIALS AND METHODS

The study published by FRECHKOP (1955) is a major source of historical information about the composition of bat populations that hibernate in Belgium's underground cavities. It describes the banding of bats (n=6,809 individuals) at 229 sites in Belgium, of which 195 are located in the Walloon Region. One of the authors (J.V.) banded a quarter of these bats.

Among these 195 sites of the Walloon Region, we did not include sites that satisfied one of the following five criteria:

- sites whose names and locations were not sufficiently precise or explicit in order to avoid ambiguity and confusion between sites.
- vast roosts where it was practically impossible to band the entire bat population (examples are Montagne-Saint-Pierre, Orp-Jauche, and Folx-les-Caves).
- roosts that were not underground sites.
- roosts that have been destroyed between the two survey periods.
- roosts for which insufficient recent census data were available, despite supplemental surveying done for the purpose of this comparison.

In light of these constraints, we were finally able to compare data for 58 roosts (Table 1). Some major current hibernation roosts for bats in the Walloon Region were not specifically indicated in the old counts. These were the underground quarries of La Malogne in Mons, Le Grand Banc at Comblain-au-Pont, La Montagne Saint-Pierre at Visé, and a few slate quarries in the Upper and Middle Ardennes.

Most of the roosts that we chose (48/58 sites) are natural karst formations. They are distributed, by natural

region, as follows (Fig. 1): 43 in Condroz and Calestienne; 9 in Famenne; 4 north of the rivers Sambre and Meuse; and one each in Ardennes and Lorraine.

The old data extracted from FRECHKOP's (1955) study consisted of the number of individuals of each species that were banded or recaptured in a roost during a winter. Even though a major banding effort was made, a very powerful light was used, and all the tricks for capturing highly inaccessible individuals were employed (VERSCHUREN, 2001), the possibility that the technique for exploring such cavities has improved over the decades cannot be ruled out (GILSON & MOËS, 1982; FAIRON, 1999). Despite under-estimation being plausible, we assumed that the number of individuals banded was considered to be the total number of observed individuals. Only later were the populations' waning numbers, then the impact of banding, and finally the need to protect these mammals (FAIRON, 1981; VERSCHUREN, 2001) ascertained.

Banding in each roost was generally conducted in multiple years. As species composition and numbers of each species varied considerably between years, we decided to use the maximum number of individuals that were banded for each species annually in each roost between 1939 and 1952. If banded and recaptured individuals were present simultaneously, both numbers were added to yield the total value used.

Recent data came from the observers network of Natagora's Plecotus Working Group and the Walloon Region's Nature and Forests Department's personnel. No individual was handled during these recent winter censuses. As for the older data, roosts where counts were conducted over several winters were described by the maximum number of individuals counted during any one of the annual censuses between 1995 and 2008. Although this way of regrouping the maxima might be seen as distorting reality, it was the only way to make a comparison that was as objective as possible.

The statistical analyses of trends concerning taxa, species richness and diversity were carried out using the Wilcoxon signed rank test. Kruskal-Wallis test was also used to test trends among ecological regions and cavity type. The other statistical analyses were performed by ANOVA-2.

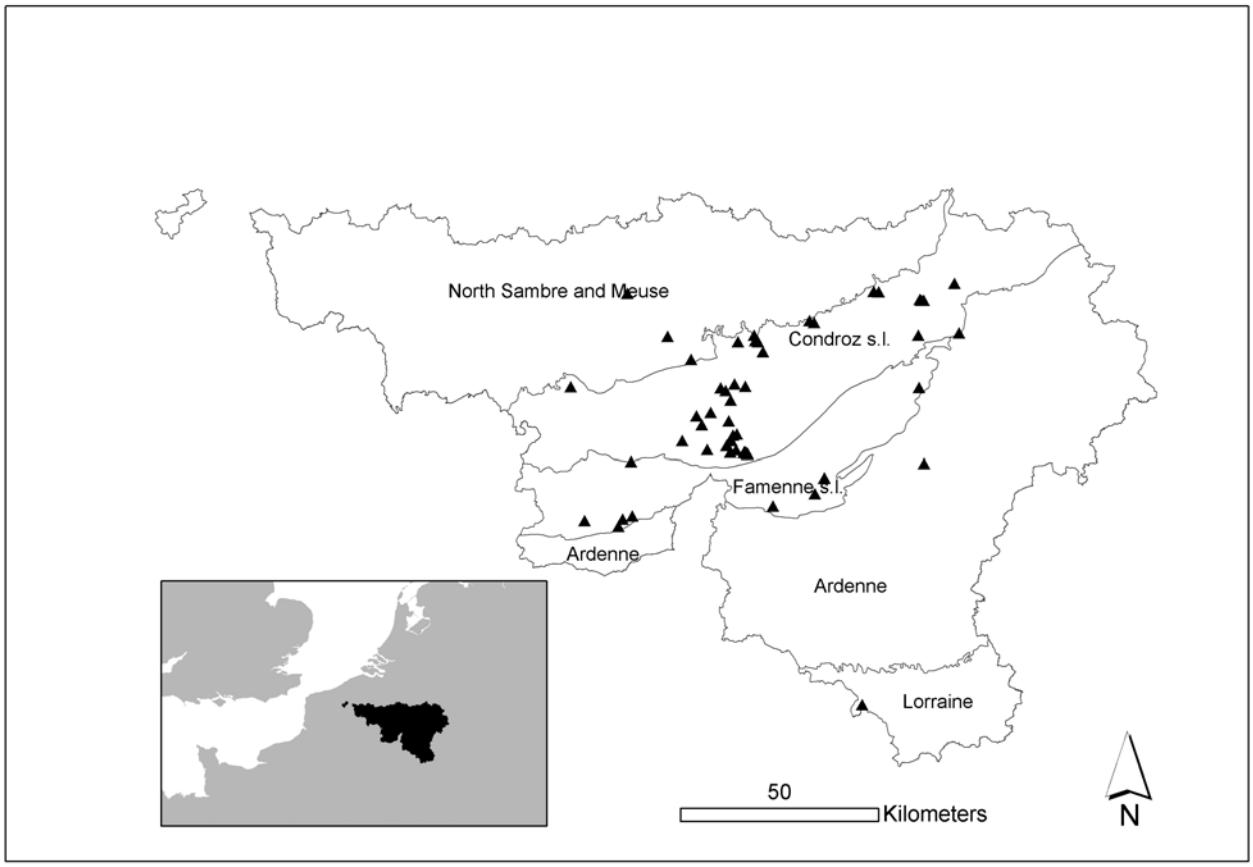

Fig. 1. – Distribution map of underground roosts inventoried in the Walloon Region.

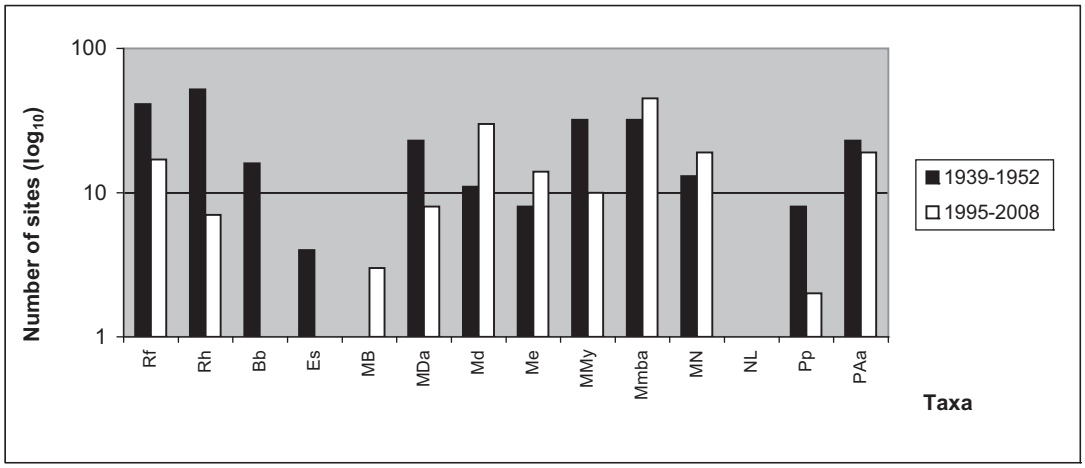

Fig. 2. – Number of roosts occupied by each taxon in 1939-1952 and 1995-2008. Taxa acronyms are explained in Table 3.

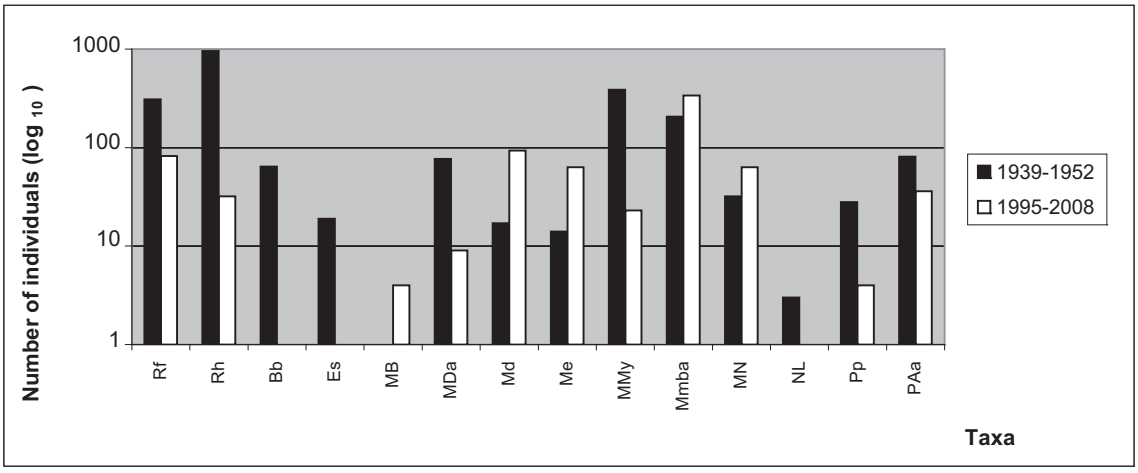

Fig. 3. – Maximum number of individuals of each taxon counted in the 58 roosts in 1939-1952 and 1995-2008. Taxa acronyms are explained in Table 3.

TABLE 1

List of the 58 underground roosts inventoried in the Walloon Region. Geographical coordinates are given in the 1972 Belgian Lambert system.

Name in Frechkop (1955)	Present name	X	Y	Ecoregion	Cavity type
Laroche en Ardenne - souterrain	Mine de Plomb à Laroche en Ardenne	236460	97910	Ardenne	artificiel
Brumagne - tunnel	Tunnel de Brumagne à Maizeret	193840	128870	Condroz s.l.	artificiel
Falaën - ruines du Château de Montaigle	Ruines du Château de Montaigle à Anhée	180310	107620	Condroz s.l.	artificiel
Flavion - trou des Nutons	Trou des Nutons à Flavion	175350	103629	Condroz s.l.	artificiel
Landelies - abbaye d'Aulne	Souterrains de l'Abbaye d'Aulne	147262	117181	Condroz s.l.	artificiel
Philippeville - souterrain	Souterrains de Philippeville	162430	98410	Condroz s.l.	artificiel
Warnant-Salet - souterrain	Carrière souterraine des Poules à Warnant-Salet	182623	110688	Condroz s.l.	artificiel
Anseremme - grotte de Colébi	Grotte Margaux à Anseremme	187520	100720	Condroz s.l.	karstique
Anseremme - grotte Moniat	Trou du Vivier à Anseremme	187779	103660	Condroz s.l.	karstique
Ben-Ahin - trou de la Truite	Trou de la Truite à Ben-Ahin (Huy)	207500	133950	Condroz s.l.	karstique
Ben-Ahin - trou du Renard	Trou du Renard à Bas-Oha (Wanze)	208660	133429	Condroz s.l.	karstique
Ben-Ahin - trou Manto	Trou Manto à Ben-Ahin (Huy)	208690	133389	Condroz s.l.	karstique

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Name in Frechkop (1955)	Present name	X	Y	Ecoregion	Cavity type
Bouvignes - Trou Madame	Trou Madame à Dinant	187110	108580	Condroz s.l.	karstique
Burnot-lez-Profondeville - Trou du Curé	Trou du Curé à Burnot-lez-Profondeville	185130	116940	Condroz s.l.	karstique
Chaleux - chantoir des Sources	Galerie des Sources à Hulsonniaux (Houyet)	191380	100730	Condroz s.l.	karstique
Chaleux - trou de la Naulette	Trou de la Naulette à Hulsonniaux (Houyet)	190950	100650	Condroz s.l.	karstique
Comblain-au-Pont - grotte	Grotte de l'Abîme à Comblain-au-Pont	235009	130250	Condroz s.l.	karstique
Dinant - grotte de	Grotte la Merveilleuse à Dinant	188180	104940	Condroz s.l.	karstique
Dinant - grotte jardin Casino	Grotte du Casino à Dinant	189160	105220	Condroz s.l.	karstique
Engihoul - grotte des végétations	Grotte aux Végétations à Yvoz-Ramet (Flémalle)	225050	141250	Condroz s.l.	karstique
Engihoul - grotte Lyell	Grotte Lyell à Ehein (Engis)	223740	141309	Condroz s.l.	karstique
Furfooz - grotte de la Gatte d'Or	Grotte de la Gatte d'Or à Furfooz (Dinant)	191880	100260	Condroz s.l.	karstique
Furfooz - puits des Vaux	Puits des Vaulx à Furfooz (Dinant)	191669	100440	Condroz s.l.	karstique
Furfooz - trou de la Machoire	Trou de la Machoire à Furfooz (Dinant)	191910	100310	Condroz s.l.	karstique
Furfooz - trou des Nutons	Trou des Nutons à Furfooz (Dinant)	191930	100420	Condroz s.l.	karstique
Furfooz - trou Louis	Trou Louis à Chaleux-Furfooz (Houyet)	191130	100800	Condroz s.l.	karstique
Furfooz - trou qui fume	Trou qui fume à Furfooz (Dinant)	191900	100440	Condroz s.l.	karstique
Godinne - grotte Chauvaux	Grotte inférieure de Chauvaux à Mont-Godinne (Yvoir)	186320	116279	Condroz s.l.	karstique
Goyet - grottes de	Grottes préhistoriques de Goyet à Mozet (Gesves)	195779	126089	Condroz s.l.	karstique
Hastière-Lavaux - grotte du pont d'Arcole	Grotte du Pont d'Arcole à Hastière	181750	101389	Condroz s.l.	karstique
Lives - souterrain	Trou de l'Eau à Lives (Namur)	189490	128540	Condroz s.l.	karstique
Lustin - trou d'Haquin	Trou d'Haquin à Maillen (Assesse)	188570	117860	Condroz s.l.	karstique
Pont-à-Lesse - trou de la Tour à Samson	Grotte Roger à Thon-Samson (Andenne)	194509	128580	Condroz s.l.	karstique
Pont-à-Lesse - Trou Magritte	Trou Magritte à Dinant	188940	101330	Condroz s.l.	karstique
Remouchamps - grotte	Grotte de Remouchamps à Sougné-Remouchamps (Aywaille)	245339	130789	Condroz s.l.	karstique
Sosoye - trou des Nutons	Trou des Nutons à Sosoye	178990	109810	Condroz s.l.	karstique
Tilff - grotte Brialmont	Grotte Brialmont à Tilff (Esneux)	236339	139080	Condroz s.l.	karstique
Tilff - grotte Dumonceau	Grotte de Monceau à Esneux	235380	139229	Condroz s.l.	karstique
Tilff - grotte Ste-Anne	Grotte Saint-Anne à Tilff (Esneux)	235840	139100	Condroz s.l.	karstique
Trooz - grotte	Grottes préhistoriques de Fond-de-Forêt à Forêt (Trooz)	244180	143310	Condroz s.l.	karstique
Waulsort - grotte de Freyr	Grotte de Freyr	186559	102349	Condroz s.l.	karstique
Waulsort - trou des Moines	Trou des Moines à Waulsort	186800	102430	Condroz s.l.	karstique
Yvoir - orphelinat	Grotte Toulemonde à Yvoir	187619	113809	Condroz s.l.	karstique
Yvoz-Mailen - trou Balza	Trou Balza à Maillen (Assesse)	191309	117319	Condroz s.l.	karstique
Bomal - Hohière, grotte	Grotte de Hohière à Heyd (Durbuy)	235229	117019	Famenne s.l.	karstique
Couvin - abîme	Trou de l'Abîme à Couvin	159305	82100	Famenne s.l.	karstique
Han s-Lesse - grottes	Grotte de Han à Han-sur-Lesse (Rochefort)	208860	90269	Famenne s.l.	karstique
Honnay - grotte de Revogne	Grotte de Revogne à Honnay (Beauraing)	198350	87230	Famenne s.l.	karstique
Lompret - grotte	Grotte de Lompret	150750	83500	Famenne s.l.	karstique
Nismes - grotte	Grotte du Pont d'Avignon à Nismes (Viroinval)	162735	84714	Famenne s.l.	karstique
Petigny - grotte de l'Adugeoir	Grotte de l'Adugeoir / grotte de Neptune à Couvin	160339	83879	Famenne s.l.	karstique
Rochefort - grotte de	Grotte touristique de Rochefort à Rochefort	211259	94169	Famenne s.l.	karstique
Rochefort - trou Maulin	Grotte du Nou-Maulin à Rochefort	211300	94300	Famenne s.l.	karstique
Orval - ruines de l 'Abbaye	Souterrains de l'Abbaye d'Orval	220851	36972	Lorraine	artificiel

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List of the 58 underground roosts inventoried in the Walloon Region. Geographical coordinates are given in the 1972 Belgian Lambert system.

Name in Frechkop (1955)	Present name	X	Y	Ecoregion	Cavity type
Marche-les-Dames - mines de fer	Galerie de Férauge à Marche-les-Dames	193520	130190	Nord sillon Sambre et Meuse	artificiel
Villers-la-Ville - abbaye	Ruines de l'abbaye à Villers-la-Ville	161520	140850	Nord sillon Sambre et Meuse	artificiel
Floreffe - grottes de	Grotte touristique de Floreffe	177602	124062	Nord sillon Sambre et Meuse	karstique
Spy - grotte de	Grotte de Spy	171699	129969	Nord sillon Sambre et Meuse	karstique

TABLE 2

Comparison of old and recent counts

	Old survey	Recent survey
Survey periods	1939-1955	1995-2008
Objective	ringing	census
Number of census	192	266
Average number of census per cavity	3.3 +/- 2.2	5.3 +/- 4.5
Number of individuals	2190	748
Number of taxa	13	12
Average species richness per cavity	4.5 +/- 2.8	3.0 +/- 2.4
Average species diversity per cavity	1.4 +/- 1.2	0.7 +/- 0.9

RESULTS

Whereas in the earlier period the 58 roosts were covered by a total of 192 censuses, the recent observations came from 266 censuses (Table 2). On average, each roost was visited 3.3 times during the earlier period compared with 5.3 times during the recent period.

The older data were composed of 2,190 banded or recaptured individuals belonging to thirteen different taxa. The recent data concerned 748 individuals belonging to twelve different taxa. One species, Bechstein's bat (*Myotis bechsteini*), was observed during the recent period only, whereas two species – *Barbastella barbastellus* and *Nyctalus leisleri* – were no longer encountered recently (Table 2). Moreover, not a single bat was found in eight of the fifty-eight roosts visited recently.

The 58 roosts that were selected cover around 11% of the hibernating bats counted annually in the Walloon Region in recent years (n=about 6,600 for 2005, SPW-DGARNE database).

The mean species richness by roost was 4.5 species during the first census period and dropped to 3.0 for the recent period ($F=9.59$, $p<0.01$; Wilcoxon signed rank test, $p<0.001$). Moreover, the species diversity index (as defined by Shannon-Weaver) fell from 1.4 to 0.70 ($F=12.71$, $p<0.01$; Wilcoxon signed rank test, $p<0.001$).

The two periods were compared by testing numbers for each taxon (Table 3) by a the Wilcoxon signed rank test, as well as species richness and diversity site by site. A bivariate non-parametric Kruskal-Wallis test was used to test potential breakdown by ecological region and type of cavity (natural versus man-made).

Changes in numbers of sites occupied and numbers of individual encountered by species before 1953 and after 1994 are shown in Figs 2 & 3. Whereas 309 *R. ferrumequinum* were banded in these roosts before 1953, only 82 specimens were observed recently.

R. hipposideros used to be the most abundant species hibernating in the underground cavities, with nearly a thousand individuals and accounting for more than 43% of the banded bats. In the recent survey, only 32 individuals were counted, which is about 3.4% of the previous number of individuals.

B. barbastellus showed an even more severe decline: a total of 64 individuals were formerly counted in the roosts studied, whereas not a single individual was found recently in these roosts.

The population of *M. dascyneme*, a migratory species that is present in Wallonia almost exclusively during the hibernation period, declined significantly: Whereas 77 individuals were banded previously, only 9 individuals were counted recently.

TABLE 3

Comparison of the number and proportion of each bat species in the two survey periods.

Species	Acronyms	total number 1939-1952	total number 1995-2008	proportion 1939-1952	proportion 1995-2008	Paired difference in median	P Wilcoxon ranked signed test	cavity type (natural vs artificial cavity)	ecoregion
Rhinolophus ferrumequinum (Schreber, 1774)	Rf	309	82	14,1%	11,0%	-3,00	P < 0,001	ns	ns
Rhinolophus hipposideros (Bechstein, 1800)	Rh	953	32	43,5%	4,3%	-10,50	P < 0,001	ns	ns
Barbastella barbastellus (Schreber, 1774)	Bb	64	0	2,9%	0,0%	-3,00	P < 0,001	ns	ns
Eptesicus serotinus (Schreber, 1774)	Es	19	1	0,9%	0,1%	-2,00	ns	ns	ns
Myotis bechsteini (Kuhl, 1817)	MB	0	4	0,0%	0,5%	1,25	ns	ns	ns
Myotis dasycneme (Boie, 1825)	MDa	77	9	3,5%	1,2%	-2,50	P < 0,001	ns	ns
Myotis daubentoni (Kuhl, 1817)	Md	17	93	0,8%	12,4%	2,00	P < 0,001	ns	ns
Myotis emarginatus (Geoffroy, 1806)	Me	14	63	0,6%	8,4%	0,75	P = 0,100	ns	ns
Myotis myotis (Borkhausen, 1797)	MMy	387	23	17,7%	3,1%	-3,00	P < 0,001	ns	ns
Myotis mystacinus (Kuhl, 1817) / *M. brandti* (Eversmann, 1845) / *M. alcathoe* Helversen & Heller, 2001	Mmba	206	338	9,4%	45,2%	2,00	P = 0,001	ns	P < 0,001
Myotis nattereri (Kuhl, 1817)	MN	32	63	1,5%	8,4%	0,50	ns	ns	ns
Nyctalus leisleri (Kuhl, 1817)	NL	3	0	0,1%	0,0%	-	ns	ns	ns
Pipistrellus sp. Kaup,1829	Pp	28	4	1,3%	0,5%	-1,50	ns	ns	ns
Plecotus auritus (Linné, 1758) / *P. austriacus* (Fischer, 1829)	PAa	81	36	3,7%	4,8%	-1,00	P < 0,05	P = 0,038	ns
Total		**2190**	**748**	**100,0%**	**100,0%**				

Twenty-three individual specimens of *M. myotis* have been seen in recent years compared with 387 in the past, and the proportion of this species in the counts has fallen off sharply, from 17.7 to 3.1%.

In contrast to these declines, numbers of some species increased significantly during the second survey period: counts of *M. daubentoni* and *M. emarginatus* increased from 70 to 93 individuals and from 14 to 63 individuals respectively.

A net rise was also observed for the number of *M. mystacinus/brandtii/alcathoe*: from 206 to 338 individuals. Whereas this species complex accounted for less than 10% of the bats that were banded in the past, it now accounts for almost half of the bats observed. This rise is not uniform across the territory and has occurred predominantly north of the Sambre and Meuse valleys.

Plecotus auritus and *P. austriacus* are declining: their abundance is halved after 50 years. Preferential occupancy of man-made cavities has been ascertained only for these taxa.

The small numbers of *M. bechsteini, M. nattereri,* and *Nyctalus leisleri* that are generally counted in the winter make it impossible to draw significant conclusions concerning their demographic change. Data about *Eptesicus serotinus* and *Pipistrellus sp*. were not taken into consideration since these taxa do not tend to hibernate in underground cavities but are found more readily in crevices in buildings.

DISCUSSION

Our comparison of the total number of bats seen in the fifty-eight roosts studied both in the past and in recent years shows that total numbers have nearly been divided by 3, despite the fact that the number of censuses made recently was higher and benefited from improved detecting techniques. In addition, species richness and diversity showed a remarkable decrease.

The availability or accessibility of hibernation roosts in the Walloon Region and contiguous regions has changed over the past fifty years. Several sites were destroyed, notably by the extension of quarries or filling of entrances, but new sites were made available thanks to the cessation of various human activities or through active

bat protection measures (LAMOTTE, 2007). Similarly, the size of the natural karst network accessible to bats increased over this period due to the activities of cavers to unblock passages, thereby creating new possibilities for hibernation in natural environments. One cannot rule out the possibility that the trends documented here result to a certain extent from a change in the availability of hibernation roosts and a redistribution of bat populations amongst them. However, this can only partially explain the trends in populations of *Chiroptera*. Otherwise, each taxon's proportion in the various roosts studied would have remained relatively stable, which is clearly not the case. What is more, the geographical comparison carried out by LAMOTTE (2007) using a much larger corpus than the data gleaned from the fifty-eight roosts analysed here does not reveal a change in the taxa's spatial distributions on the regional scale that could explain the variations seen locally.

This study confirms, quantitatively and on the regional scale, the findings of a number of other studies on this subject. Quantitative comparison between these old and recent data shows, for many species, major declines in numbers of individuals in persisting hibernation roosts. The species concerned in particular by this trend are *B. barbastellus, R. ferrumequinum, R. hipposideros, M. myotis,* and *M. dasycneme*.

In addition to the harmful consequences of disturbing hibernating individuals as a result of human activities, such as underground exploration and/or touristic visits (FAIRON, 1967; SPEAKMAN & RACEY, 1989; SPEAKMAN et al., 1991; THOMAS, 1995), including the banding of the specimens, many other factors have been suggested to explain the declines in the populations.

Apart from hibernacula disturbance, many other factors presumably impact resident bat populations: road traffic collision (LEMAIRE & ARTHUR, 1998), nocturnal light (ARLETTAZ et al., 2000; RICH & LONGCORE, 2005), decline of major insect prey species such as cockchafers for instance, disturbance in summer roosts.

R. ferrumequinum, for example, demands summer roost requirements. Its decline, which has also been underlined by FAIRON (1997), can be linked to the decrease of its preferred prey species such as *Aphodius rufipes* dung beetles, cockchafers, tipulids (RANSOME & HUTSON, 2000) in the Walloon Region (DELAHAYE & KERVYN, 2001). Antiparasitic treatment of cattle presumably negatively affects the abundance of *Aphodius rufipes* beetles, the only prey species upon which the greater horseshoe bat feeds in late summer (RANSOME & HUTSON, 2000). A negative impact of banding also needs to be considered for this species (VERSCHUREN, 2001; DIETZ et al., 2006).

Like *R. ferrumequinum, R. hipposideros* is particularly vulnerable when hibernating, given its hanging position from the ceiling of its roosts. FAIRON (1977) had already underlined some forty years ago *R. hipposideros's* sensitivity to recapture throughout the banding period and the mortality that could ensue. What is more, given its hunting strategy, which consists in flying constantly under the forest canopy or under the cover of hedges, *R. hipposideros* coped with a drastic reduction in the accessibility of its hunting grounds due to the destruction of the network of hedges and tree alignments in the Walloon landscape (MOTTE & LIBOIS, 2002; SCHOFIELD, 2008). Further research needs to be carried out on the impact of pesticide contamination and light pollution on this species (BONTADINA et al., 2008). The latter could play a major role in food competition with the commonest species in Europe, the pipistrelle bat (ARLETTAZ et al., 2000).

The reasons *B. barbastellus* has almost disappeared from the Walloon Region (FAIRON & BUSCH, 2003) have not yet been elucidated. Nevertheless, its great sensitivity to disturbance, even in its forest summer roosts (RUSSO et al., 2004), must be underlined, as well as this species' highly selective diet (RYDELL et al., 1996; SIERRO & ARLETTAZ, 1997; SIERRO, 2003).

Causes for *M. dasycneme*'s decline have not yet been identified with certainty, although major changes in hunting habitats and a drop in the number of optimal summer roosts are suspected (LIMPENS et al., 2000).

The apparent increase of *M. emarginatus* could be attributed to methodological issues. It is plausible that banders previously under-estimated its presence because of its habit of clustering at the highest points of the ceilings in the underground cavities where it hibernates, i.e. where temperature is slightly higher. Given the small number inventoried, no clear statements about the evolution of the numbers of this species can be made.

Decline in the *M. myotis* population is most likely also due to the decline of some of its identified key prey species in the Walloon Region (KERVYN, 1996;). Limited accessibility to the vast summer roosts required by this species likely had an impact as well and is probably more important than that of the availability of optimal hunting grounds (GÜTTINGER, 1997). However, the detection of prey on its hunting grounds by this ground-gleaning bat could be hindered by noise, traffic noise especially (SCHAUB et al., 2008).

The decline of long-eared bats (*P. auritus / P. austriacus*) is probably also related to reduced roosts and prey availability. Further research needs to be carried on these species in order to monitor whether these two sibling species have similar ecological requirements and conservation status.

The taxa whose numbers apparently increased, i.e *M. daubentoni* and *M. mystacinus/brandtii/alcathoe*, are remarkable given that, over the past fifty years, they have become the most frequently observed taxa in underground cavities during winter. The possibility that the apparent increases in these taxa – which tend to hibernate in deep, narrow crevices – result from improved census techniques cannot be ruled out (FAIRON, 1999). Improved diagnostic criteria could also explain the increased abundance of *M. daubentoni* in recent counts (GILSON & MOËS, 1982). Finally, the rise in the *M. daubentoni* population could be linked to eutrophication of aquatic habitats (WARREN et al., 2000), which is likely to increase the availability of *Chironomidae* that are consumed in great quantities by this species (BECK, 1995).

CONCLUSIONS

This long-term comparison of hibernating bat populations shows major changes that occurred in the composition of the bat community in Wallonia. The populations of five species have declined, whereas those of two others have increased. The fifty percent loss of species diversity observed in this study is both a reflection of the far-reaching ecosystem deterioration in the Walloon Region and the source of considerable change in the way these ecosystems function.

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Interspecific morphometric variation in the postcranial skeleton in the genus *Apodemus*

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ABSTRACT. Wood mice (genus *Apodemus*) are common murid rodents in the Palearctic region. In spite of the fact that they exhibit high phenotypic similarity, individual species (populations) differ in their preferred habitat (woodlands, steppes-fields, rocks) and behaviour (tendency to digging, jumping, climbing). It is therefore of special interest to evaluate interspecific (inter-population) variability in postcranial skeleton within this group and to suggest ecological interpretations of observed differences. We studied skeletons of 265 wood mice belonging to seven species from Europe and the Middle East: *Apodemus agrarius* (subgenus *Apodemus*), *A. mystacinus* (subgenus *Karstomys*), *A. hyrcanicus*, *A. witherbyi*, *A. uralensis* (=*microps*), *A. flavicollis* and *A. sylvaticus* (subgenus *Sylvaemus*). Thirty five postcranial and body measurements were obtained and analysed using multivariate statistics. The multivariate analysis, based on size adjusted data, revealed clear morphological separation among species belonging to different subgenera. The morphological characters responsible for this separation and the position of the control sample of *A. peninsulae* (belongs to the same subgenus as *A. agrarius*, but differs in preferred habitat) in morphospace support the view, that ecology participated in the shaping of the postcranial skeleton of the studied species. *A. agrarius* possesses the characters associated with digging activity, *A. mystacinus* with jumping and *Sylvaemus* species with fast terrestrial movement and climbing. However, there were found only subtle morphological differences among individual *Sylvaemus* species, in spite of variability in their ecological requirements.

KEY WORDS : morphometrics, ecomorphology, wood mice, *Sylvaemus, Apodemus*, *Karstomys*

INTRODUCTION

Muroid rodents (family Muridae) represent a highly diversified mammalian clade inhabiting nearly all habitat types. Consequently, individual rodent species exhibit a suite of characteristics that are associated with a particular lifestyle. These may comprise behaviour (adaptive profile: DEWSBURY et al., 1982), locomotor performance (*sensu* GARLAND, 1994) and morphology (e.g. PRICE, 1993). Closely related rodent taxa exhibiting different ecological and behavioural strategies, such as wood mice of the genus *Apodemus* Kaup, 1829, may serve as an appropriate model for understanding the evolution of species-specific design.

Wood mice are common murid rodents in the Palaearctic region (cf. MUSSER et al., 1996; MITCHELL-JONES et al., 1999) where they fill the same adaptive zone as the genus *Peromyscus* in North America (MONTGOMERY, 1989). All *Apodemus* species are opportunistic seed eaters that also consume insects and diverse additional vegetable components (e.g. MIRIĆ, 1966; HOLIŠOVÁ, 1967; HOLIŠOVÁ & OBRTEL, 1977; 1980; BABINSKA-WERKA, 1981; OBRTEL & HOLIŠOVÁ, 1983; GEBCZYNSKA et al., 1987; MONTGOMERY & MONTGOMERY, 1990; HEROLDOVÁ, 1994; ROGERS & GORMAN, 1995). They share generalized muroid morphology and exhibit high phenotypic similarity among species (e.g. FRYNTA et al., 2006), but individual *Apodemus* species/populations differ in their preferred habitats and behaviour.

Species of the genus *Apodemus* inhabiting Europe, North Africa and Western Asia form three distinct clades (MUSSER et al., 1996; for genetic support see e.g. MARTIN et al., 2000; MICHAUX et al., 2002; BELLINVIA, 2004) corresponding to traditionally recognized subgenera: *Apodemus*, *Karstomys* Martino, 1939 and *Sylvaemus* Ognev, 1924.

Apodemus agrarius (Pallas, 1771), belonging to the East Asian subgenus *Apodemus,* is least related to the other wood mice species of the western Palaearctics. It has only recently (early Holocene) extended its range from the Far East westwards to Europe (BÖHME, 1978). *A. agrarius* is predominantly field-dwelling and associated with crop-fields, grasslands, and open wet habitats, especially along rivers and streams (KRATOCHVÍL, 1962; 1977; ZEJDA, 1967; KARASEVA et al., 1992).

The subgenus *Karstomys* consists of only two species: *A. epimelas* (Nehring, 1902) from the Balkans and *A. mystacinus* (Danford and Alston, 1877) from the Island of Crete and the Middle East (see VOHRALÍK et al., 2002). Both of these species are specialised rock-dwellers and represent the largest forms of the genus *Apodemus*. They do not make their own burrows like other studied *Apodemus* species, instead using rock cavities as nests (MIRIĆ, 1966; GROLL, 1992).

Subgenus *Sylvaemus* contains at least six species. Three of them, *A. flavicollis* (Melchior, 1834)*, A. sylvaticus* (Linnaeus, 1758) and *A. uralensis* (Pallas, 1811), including *A. microps* Kratochvíl and Rosický, 1952, are traditionally recognised and represent the most morphologically differentiated forms of the subgenus (STEINER, 1968; FRYNTA et al., 2006). In Central Europe, they exhibit contrasting ecological strategies. *A. flavicollis* is

a forest-dweller (e.g. STEINER, 1968; MONTGOMERY, 1977; MARSH & HARRIS, 2000), *A. uralensis* a field-dweller (e.g. KRATOCHVÍL, 1962; STANKO, 1994), and *A. sylvaticus* exhibits less specialised requirements, reaching its maximal abundance in ecotones including forest margins, bushes, set aside fields, parks, etc (e.g. ZEJDA, 1965; STEINER, 1968; ČIHÁKOVÁ et al., 1993; FRYNTA et al., 1994). These preferences were clearly supported by a study of rodent assemblages in windbreaks and adjacent fields performed in southern Moravia (Central Europe; PELIKÁN, 1986). Moreover, *A. flavicollis* and *A. sylvaticus* are known to exhibit considerable arboreal activity (*A. flavicollis*: BOROWSKI, 1962; HOLIŠOVÁ, 1969; MONTGOMERY, 1980; JUŠKAITIS, 1995; *A. sylvaticus*: MONTGOMERY, 1980; SANTOS & TELLERÍA, 1991; TATTERSALL & WHITBREAD, 1994 and references therein). Both species are able to use tree cavities instead of subterranean nests.

While the habitat requirements of European species have been studied in detail (see above), only fragmentary information is available for the *Sylvaemus* species of the Middle East. *A. hyrcanicus* Voronstov, Boyeskorov, Mezhzherin, Lyapunova, and Kandaurov, 1992, only recently recognised form from the Hyrcanian area along the Caspian Sea, is obviously confined to forest (our data, VORONTSOV et al., 1992). Populations of the other species : *A. uralensis* (limited to the Northern Anatolia and Transcaucasus), *A. flavicollis*, and *A. witherbyi* (Thomas, 1902), may be found syntopically. Nevertheless, *A. witherbyi* is the only species of this area regularly inhabiting steppes and/or semideserts, while the former two species are more or less restricted to forest and bushes (FILIPPUCCI et al., 1989; FILIPPUCCI et al., 1996; MACHOLÁN et al., 2001; and our unpublished data).

The different habitat preferences described above may be associated with different locomotor performance of particular *Apodemus* species and possibly adaptive evolution of relevant morphological traits. We can assume that species living in open microhabitats (including forest/shrub habitats without dense undercover) should possess morphological traits associated with fast running and jumping. Species inhabiting forest habitats should possess morphological traits associated with climbing. Species using subterranean nests should possess morphological traits associated with digging.

Unfortunately, there is only limited information concerning the locomotor performance and morphology of individual *Apodemus* species. When subjected to ten minute laboratory tests for exploratory behaviour (FRYNTA, 1992; 1994), the *Apodemus* fall into three groups corresponding to subgenera. Among seven *Apodemus* species/subspecies included in this study, *A. epimelas* (the closest relative of *A. mystacinus)* exhibited the highest activity, while the representatives of the subgenus *Apodemus*, especially the European population of *A. agrarius,* had the lowest activity. The species of the subgenus *Sylvaemus* have a fairly intermediate position. Jumping was correlated with activity scores (FRYNTA, 1994). This behaviour has never been recorded in a European population of *A. agrarius* during the experiments. It was rare in *A. uralensis* (mean=0.4 jumps per 10min test), and frequent in *A. flavicollis* (2.7), *A. sylvaticus* (4.0) and *A. epimelas* (7.9).

Considerable research effort, mostly for taxonomical and determination purposes, has been devoted to morphometric differences among *Apodemus* species (e.g. FILIPPUCCI et al., 1984; POPOV, 1993; PANZIRONI et al., 1994; LAVRENCHENKO & LIKHNOVA, 1995; ÖZKAN & KRYŠTUFEK, 1999; REUTTER et al., 1999; FRYNTA et al., 2001). Therefore, the authors focused on cranial measurements that are usually supposed to be less affected by adaptive evolution. Recently we have analysed multivariate cranial morphometry of nine *Apodemus* species (16 samples, FRYNTA et al., 2006) and found a good correspondence between our phenetic tree and the current phylogenetic hypothesis based on DNA sequences (MICHAUX et al., 2002; BELLINVIA, 2004).

In contrast, limited information is available about the morphological traits of *Apodemus* species that may be associated with their type of locomotion. Attention to date has focused on some external measurements. The lengths of the tail and the hind-foot and the eye diameter have traditionally been considered by field workers to distinguish among the European *Sylvaemus* species of similar appearance, whereas the small eye diameter and the short tail and hind-foot in *A. uralensis* (as well as in *A. agrarius* of the subgenus *Apodemus)* are supposed to be attributed to the high proportion of activity in burrows in this species (HOLIŠOVÁ et al., 1962; NIETHAMMER & KRAPP, 1978). Similarly, the length of vibrissae is expected to be functionally related to the diameter of investigated space. The subterranean species usually have short vibrissae, while those of rock-dwelling (petricolous) species are extremely long. KRATOCHVÍL (1968) described vibrissae in five *Apodemus* species and found that their length increases sharply in the following order: *A. agrarius, A. uralensis, A. sylvaticus, A. flavicollis* and *A. mystacinus.*

To be able to explain the interspecific variation in morphology found within *Apodemus,* we need to make use of the functional interpretation of the characters and the relationship between morphology and locomotor performance reported within other taxa. In this respect most studies are devoted to studying morphological adaptations for a subterranean mode of life. These adaptations are mostly associated with digging activity of animals and comprise skeletal characters participating in: 1) strengthening of the forelimb skeleton (short and stout bones), 2) changes of size of areas for muscular attachments on bones (e.g. enlarged medial and lateral epicondyle of humerus, deltoid process of humerus, teres major process and acromion process of scapula) and 3) changes of position (increased ratio of in-lever arm to out-lever arm by e.g. distal position of deltoid process on humerus, elongated olecranon on ulna) of areas for muscular attachments on bones (e.g. HERÁŇ, 1962a; HILDEBRAND, 1985; NEVO, 1999; FERNÁNDES et al., 2000; STEIN, 2000; ELISSAMBURU & VIZCAÍNO, 2004; LAGARIA & YOULATOS, 2006; SAMUELS & VAN VALKENBURGH, 2008; unpublished data[1]). Similar but

[1] WARBURTON NM (1993). Functional morphology and evolution of marsupials moles (Marsupialia, Notoryctemorphia). MSc. thesis. The University of Western Australia, Perth: 1-237.

less prominent modifications are reported also for the hind limbs (short and robust long bones, enlarged epicondyle of femur, elongated tibial tuberosity, short tarsal and metatarsal bones, e.g. REED, 1951; STEIN, 2000; ELISSAMBURU & VIZCAÍNO, 2004; SAMUELS & VAN VALKENBURGH, 2008; unpublished data[1]), pelvis and axial skeleton (reduced pelvis fused to the sacrum, acetabulum shifted to the spinal axis, long ischium with massive ischial tuberosity, massive wings of ilium, elongated sacrum with its widened cranial part, short lumbar part of spinal axis e.g. HERÁŇ, 1962a; 1962b; SCHICH, 1971; NEVO, 1999; STEIN, 2000), which participate in soil removal from burrow systems and bracing the body against tunnel walls. Besides adaptations to digging there are also characters associated with movement in a narrow burrow system (short tail, short ears, short limbs, e.g. HERÁŇ, 1961; HERÁŇ, 1962a, 1992; BÖHME, 1978; NEVO, 1999; STEIN, 2000). Unfortunately, there is little information concerning adaptations associated with other types of activities observed in *Apodemus* species. It includes adaptations on limbs and vertebral column associated with arboreal activity (elongated and gracile limbs, short olecranon on ulna, loose femoral head, long lumbar part of vertebral column, broad cranial part of sacrum, long tail, e.g. DOBRORUKA, 1960; HERÁŇ, 1961; HERÁŇ, 1962a; SCHICH, 1971; POLK et al., 2000; SAMUELS & VAN VALKENBURGH, 2008) and fast terrestrial movement (short distal extension of greater trochanter of femur, long metatarsal bones, long lumbar part of vertebral column, caudal shift of acetabulum of pelvis, e.g. HERÁŇ, 1962b; SCHICH, 1971; ELISSAMBURU & VIZCAÍNO, 2004). There are also studies that deal with inner construction of bones as e.g. the amount and distribution of cortical bone in respect to ecology of studied species (BIKNEVICIUS, 1993).

This paper is focused on using postcranial skeleton measurements to advance the poorly-studied field of *Apodemus* morphology. These traits are expected to be functionally associated with locomotor performance and therefore good candidates for adaptive evolution. The aims of our study are to (1) analyze morphometric variation of the postcranial skeleton in the majority of *Apodemus* species of the Western Palaearctics, (2) compare morphometric results and available phylogenetic relationships, (3) interpret morphometric patterns in view of the ecological requirements of studied species.

MATERIALS AND METHODS

Studied specimens were collected by the authors and their colleagues during field studies in the Czech Republic and Czech expeditions to the Middle East and Far East. All specimens are deposited in the collections of the Department of Zoology, Charles University in Prague. The studied mice were captured in the field or they were of the first captive-born generation. Some of the individuals (captured in the field as well as captive-born) were kept in captivity usually for several months in order to reach their asymptotic size, others were selected according to their molar abrasion (mostly category 4 and 5 *sensu* STEINER (1968)) and can be considered as fully grown (see FRYNTA & ŽIŽKOVÁ, 1992 for the characteristic of postnatal growth in *A. sylvaticus*). The only exception was the sample of *A. peninsulae* where age separation of the individuals was not used in size-free data (see below) due to a very small sample size and that, therefore, contains also two young individuals (molar abrasion of category 2). This procedure enabled us to rule out the effect of growth (except *A. peninsulae*) while the size component of the variation remained unchanged. We studied 265 specimens belonging to the seven species – *A. flavicollis*, *A. witherbyi*, *A.* cf. *hyrcanicus*, *A. sylvaticus*, *A. uralensis*, *A. mystacinus*, *A. agrarius*. Moreover we use 7 specimens of *A. peninsulae* as a control sample. For details of individual samples (localities, sample size) see Appendix 1.

Most of the studied *Sylvaemus* specimens from the Middle East were determined by biochemical methods (allozymes, 69 specimens, MACHOLÁN et al., 2001) or they were descendants of biochemically determined individuals (11 specimens). Specimens from Sirbasan, Now Kandeh and Asalem were regarded as one *Apodemus* species, because all biochemically determined individuals from these localities belong to only one species. The remaining 18 specimens from the Middle East were identified according to Canonical Variates Analysis based on skull and body measurements (FRYNTA et al., 2001).

Four standard external measurements of each individual were taken using callipers. Subsequently the skeletons were removed and biologically prepared using *Dermestes* larvae. Thirty one postcranial measurements were taken using callipers or a stereomicroscope (see Table 1 for detailed description of the measurements and Appendix 2 for their standard descriptive statistics). To avoid repeated use of the same measurements in our analyses, LTP, LU, LH, LP, LH were used to obtain the following measurements: LTP1 (LTP minus MET) - length of tarsal bones and phalanges, LH2 (LH minus LH1) – length of distal part of humerus, LU1 (LU minus OLE)- functional length of ulna, LP1 (LP minus LP2) - length of ilium (including acetabulum), LT1 (LT minus LT2) - length of proximal part of tibia (to the fusion of tibia and fibula). See Fig. 1 for depiction of the measurements used in our analyses.

TABLE 1

List of measured postcranial characters. C – callipers, SM - stereomicroscope

	measured character	symbol	instrument	accuracy (mm)
external	length of body	LC	C	1
	length of tail	LCD	C	1
	lenght of hind foot	LTP	C	0.1
	length of ear	LA	C	0.1
skeletal - forelimb	length of humerus	LH	C	0.1
	length of proximal part of humerus (to deltoid process)	LH1	SM	0.1
	width of proximal part of humerus (including deltoid process)	WH1	SM	0.025
	width of distal part of humerus	WH2	SM	0.025
	width of distal part of humerus (laterale epicondyle – mediale epicondyle)	WH3	SM	0.05
	length of ulna	LU	C	0.1
	length of olecranon (to semilunar notch)	OLE	SM	0.05
	width of proximal part of ulna and radius	WU	SM	0.025
- scapula	length of scapula	LS	SM	0.1
	width of scapula (perpendicular to long axis of spine)	WS1	SM	0.1
	width of scapula (medial angle – inferior angle distance)	WS2	SM	0.05
- hindlimb	length of femur	LF	C	0.1
	width of proximal part of femur (including third trochanter)	WF1	SM	0.025
	width of distal part of femur	WF2	SM	0.025
	width of femoral neck	WF3	SM	0.025
	distance between greater trochanter of femur and femoral head	WF4	SM	0.025
	length of tibia	LT	C	0.1
	length of distal part of tibia (from fusion of tibia with fibula)	LT2	SM	0.1
	width of proximal part of tibia (on tibial crest)	WT1	SM	0.025
	width of distal part of tibia and fibula	WT2	SM	0.025
	length of third metatarsal bone	MET	SM	0.1
- pelvis	length of coxal bone	LP	SM	0.1
	length of ischiopubis	LP2	SM	0.05
	width of ischiopubis	WP1	SM	0.1
	width of ilium	WP2	SM	0.025
	length of obturator foramen	LSF	SM	0.05
	width of obturator foramen	WSF	SM	0.05
	distance between coxal bones	SW1	SM	0.05
	width of sacrum	SW2	SM	0.05
- backbone	length of sixth lumbar vertebra	VBL	SM	0.05
	width of sixth lumbar vertebra (on transverse processes)	VBW	SM	0.05

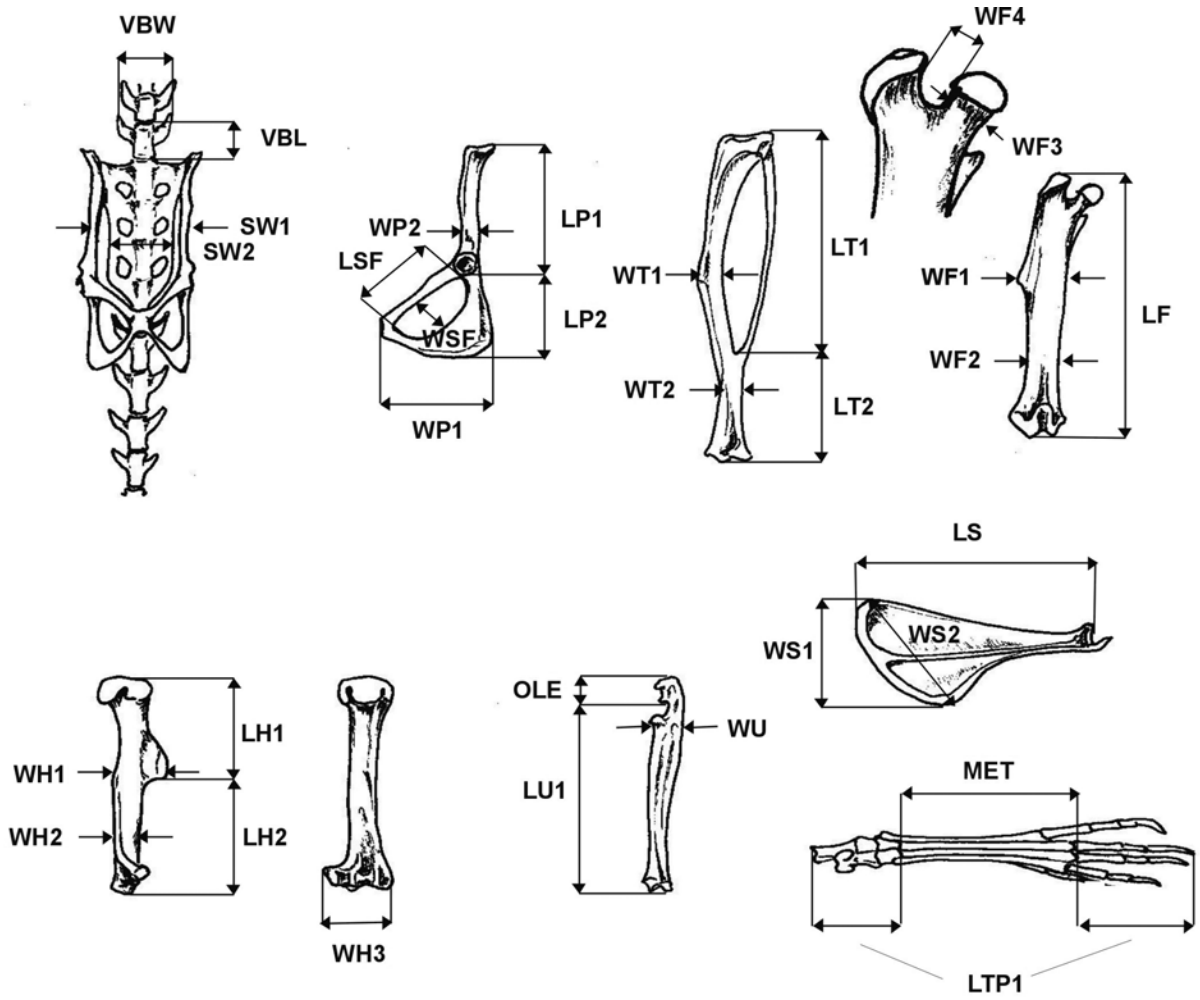

Fig. 1. – Postcranial measurements used in analyses. See Material and Methods and Table 1 for explanation of the measurement abbreviations.

The STATISTICA Analysis System (release 6.0) was used for most calculations. The data were checked for normality prior to statistical analyses. Deviations from normality were small, and most distributions were both unimodal and symmetrical as required for the multivariate procedures used here.

The data were log-transformed and missing postcranial values (in case of damaged skeletons) were replaced by those predicted from regression using most correlated variable as an independent factor (assessed according to correlation matrix of all variables). Each population was treated separately to avoid possible differences in allometries. To rule out the effect of growth and size, the Mosimann method of size adjustment (MOSIMANN, 1970) was used in Canonical Variates Analysis (CVA, see below). This data set is therefore referred to as "size-free". WS1 was omitted in size-free analyses according to the software requirements.

We visually inspected plots of log geometric mean scores (body size) vs. canonical variates scores (CV1-CV3) to detect possible hidden effects of allometries on CVA results. We found consistent allometric relationship for neither within-species nor between-species data. The only partial exception was the case of CV3 scores exhibiting a tendency to positive allometry in between-species comparison.

The log-transformed data were analysed using Principal Component Analysis (PCA). Principal component scores of the first principal component (PC1) extracted for each individual were subjected to ANOVA in order to evaluate the variation amongst the studied samples.

Size-free data were used for computing squared Mahalanobis distances (under the CVA subroutine of the STATISTICA Analysis System) between all 10 *Apodemus* samples. UPGMA clustering (STATISTICA Analysis System) was then used to construct a phenetic tree.

Next, the size-free data for 9 *Apodemus* samples (excluding *A. peninsulae* due to small sample size) were subjected to Canonical Variates Analysis. Scores of the first three canonical roots were used to visualise morphometric relationships between samples in a bivariate plot.

Classification function resulting from CVA analysis of studied samples was applied to individuals of *A. peninsulae* and computed scores of the first three canonical roots were used to visualise their position in morphospace according to other studied samples.

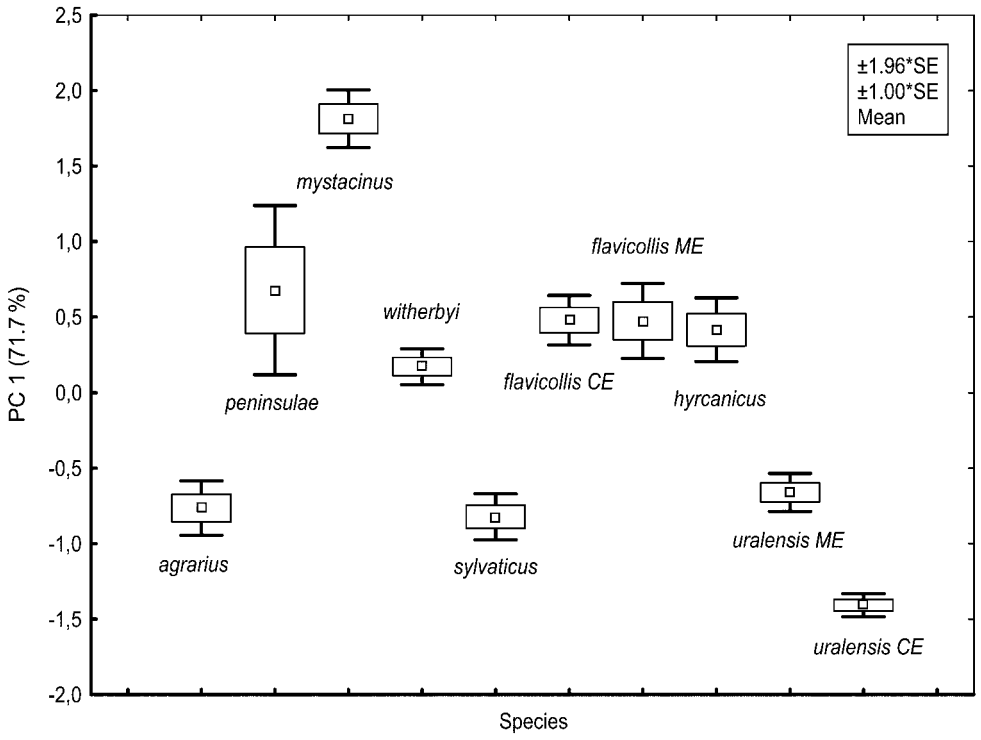

Fig. 2. – Box plots of PC 1 scores derived from original log-transformed data. CE - Central Europe, ME - the Middele East.

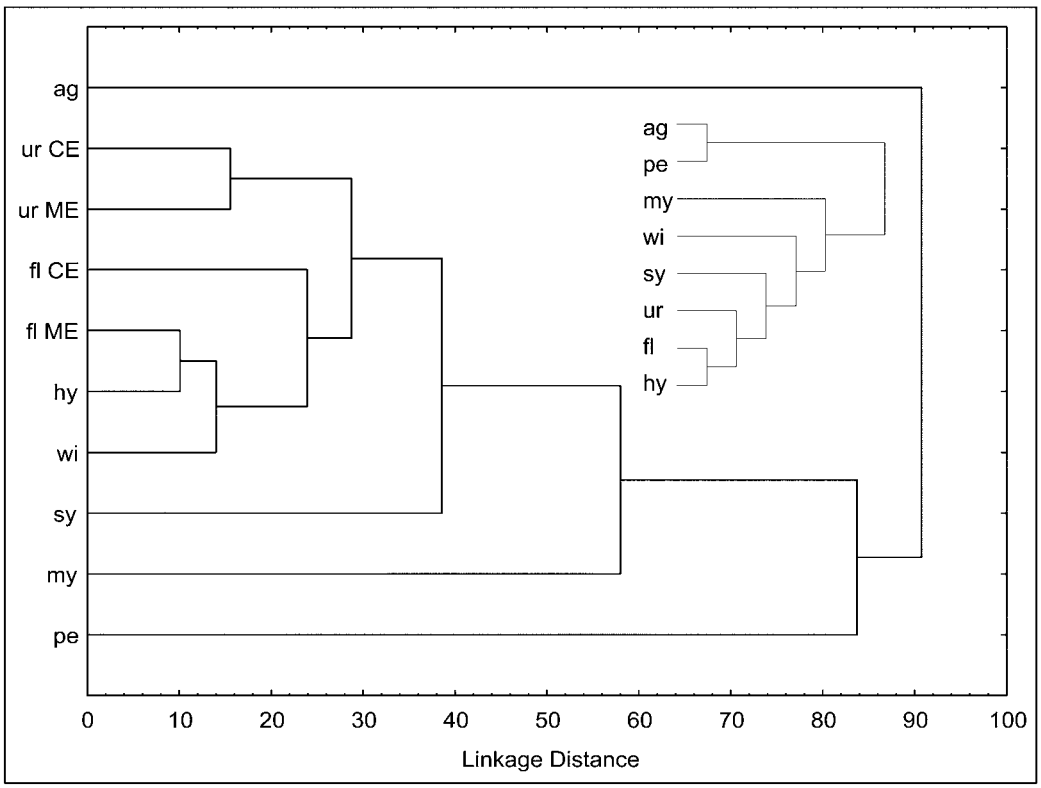

Fig. 3. – Phenetic tree from UPGMA cluster analysis, based on Mahalanobis distances computed from data adjusted by the Mosimann method (size-free data). Genetic tree derived from MICHAUX et al. (2002) and BELLINVIA (2004) in the right upper corner. CE - Central Europe, ME - the Middle East.

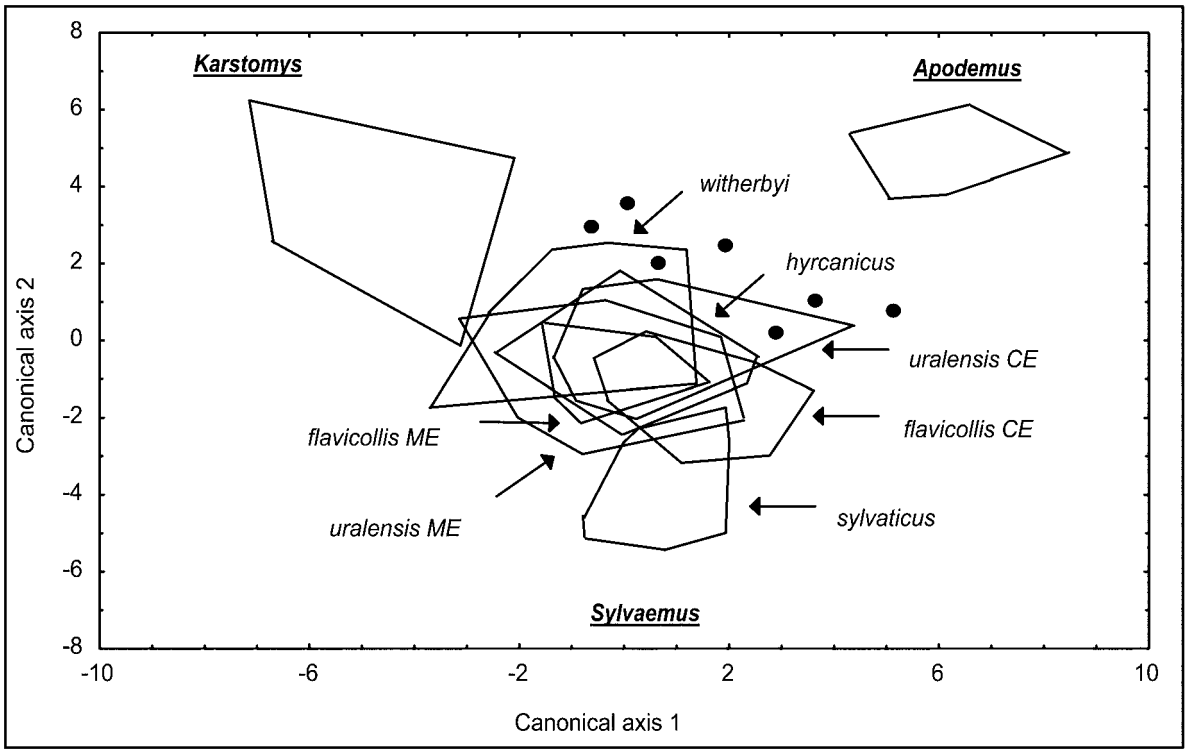

Fig. 4. – Projection of nine studied samples of *Apodemus* species onto the first two canonical variates as derived from data adjusted using the Mosimann method (size-free data). Solid circles depict position of *A. peninsulae* according to classification function resulting from Canonical variates analyses of studied samples. CE - Central Europe, ME - the Middle East.

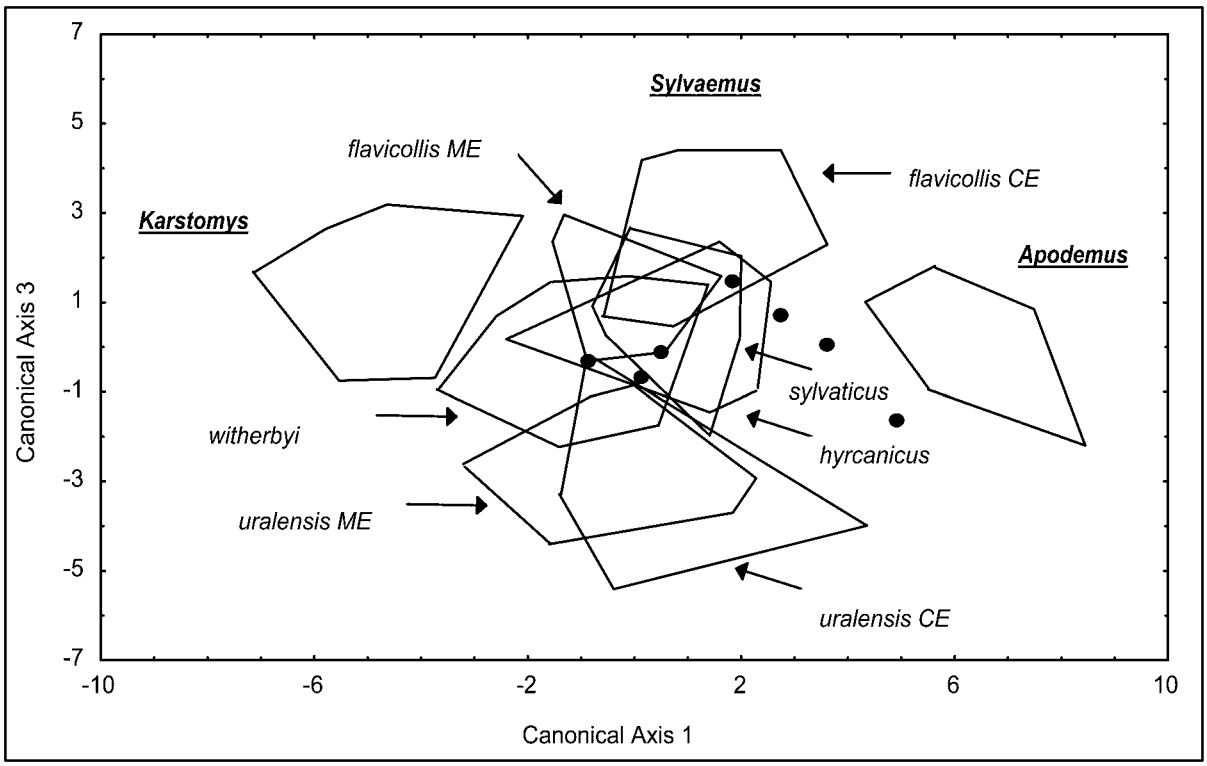

Fig. 5. – Projection of nine studied samples of *Apodemus* species onto the first and third canonical variates as derived from data adjusted using the Mosimann method (size-free data). Solid circles depict position of *A. peninsulae* according to classification function resulting from Canonical variates analyses of studied samples. CE - Central Europe, ME - the Middle East.

TABLE 2

PC 1 loadings for 35 body and postcranial measurements. Analysis based on original log-transformed data. See Material and Methods and Table 1 for measurement abbreviations.

	PC1
LC	0.847
LCD	0.853
LTP1	0.857
LA	0.709
LT2	0.783
LT1	0.918
WT1	0.782
WT2	0.886
LF	0.969
WF1	0.896
WF2	0.894
LP2	0.937
LP1	0.867
WP1	0.866
WP2	0.178
LSF	0.797
WSF	0.817
SW1	0.895
SW2	0.859
VBW	0.884
VBL	0.744
WU	0.814
WH1	0.864
WH2	0.859
LS	0.945
WS1	0.879
WS2	0.889
LU1	0.922
LH2	0.873
OLE	0.868
LH1	0.902
WH3	0.908
WF4	0.700
WF3	0.877
MET	0.745

RESULTS

PCA of log-transformed data yielded PC 1 (for loadings see Table 2), which explained 71.7% of the variance ($F=120$, $p<0.001$). It was highly and positively correlated with all traits studied and can be considered as a measure of body size. Along the PC 1 axis, the species studied split into "large" (*A. mystacinus*), "medium" (*A. peninsulae*, *A. flavicollis* from both regions*, A. hyrcanicus, A. witherbyi*), and "small" groups (*A. uralensis* from the Middle East*, A. agrarius, A. sylvaticus*)*; A. uralensis* from central Europe being the smallest one (Fig. 2).

Phenetic comparisons of size-free data (see Appendix 3 for matrix of squared Mahalanobis distances, and Fig. 3 for UPGMA tree) clearly differentiate *A. (Apodemus) agrarius* (with most basal position on phenetic tree), *A. (Apodemus) peninsulae*, *A. (Karstomys) mystacinus* and *A. (Sylvaemus) sylvaticus* (the subsequent branches) from the group of remaining species/populations of the subgenus *Sylvaemus.* Within the latter group, *A. uralensis* and European *A. flavicollis* were the most differentiated, while the samples from the Middle East populations of *A. witherbyi, A. hyrcanicus* and *A. flavicollis* clustered together.

TABLE 3

Canonical variate loadings for 34 body and postcranial measurements. Analysis based on data adjusted by Mosimann method (size-free data). See Material and Methods and Table 1 for measurement abbreviations.

	Axis 1	Axis 2	Axis 3
LC	0.209	-0.077	-0.154
LCD	-0.351	-0.059	0.016
LTP1	0.009	-0.266	0.133
LA	-0.186	-0.378	0.041
LT2	-0.207	-0.270	-0.042
LT1	-0.168	-0.153	-0.236
WT1	0.210	0.130	0.061
WT2	0.064	0.043	0.206
LF	-0.148	-0.110	0.018
WF1	-0.061	0.125	0.227
WF2	-0.046	0.142	0.150
LP2	-0.077	0.226	0.116
LP1	0.155	0.160	-0.047
WP1	-0.007	0.169	-0.139
WP2	0.308	-0.197	-0.064
LSF	0.061	0.157	-0.034
WSF	-0.099	0.358	-0.082
SW1	0.077	-0.105	0.176
SW2	-0.045	0.033	0.059
VBW	-0.097	-0.076	0.246
VBL	0.189	0.128	-0.380
OLE	0.086	0.133	0.047
LU1	-0.031	-0.299	-0.172
WU	0.178	-0.034	-0.124
LH1	-0.058	-0.011	-0.072
LH2	0.026	-0.173	-0.043
WH1	0.144	0.018	-0.082
WH2	-0.152	0.158	-0.120
LS	0.106	0.033	-0.189
WS2	0.107	-0.014	-0.271
WH3	-0.073	0.028	-0.101
WF4	-0.002	0.128	0.264
WF3	-0.183	-0.067	0.178
MET	-0.054	-0.505	-0.181

We performed CVA on size-free data (Wilks' Lambda=0.00022) in order to evaluate morphological relationships among studied samples (for this analysis the smallest sample, i.e. *A. peninsulae,* was excluded). In total 95% of specimens were correctly classified (the classification was 100% successful in specimens of *A. agrarius*, *A. mystacinus*, *A. sylvaticus* and *A. uralensis* CE; 3 specimens of *A. uralensis* ME were incorrectly classified as *A. uralensis* CE, *A. sylvaticus* and *A. wither-*

byi, 2 specimens of *A. flavicollis* CE as *A. hyrcanicus*, 2 specimens of *A. flavicolis* ME as *A. witherbyi* and 3 specimens of *A. hyrcanicus* as *A. whitherbyi*, *A. flavicollis* ME and *A. uralensis* ME). The positions of individual samples in a morphospace of the first three canonical roots are provided in Fig. 4 and Fig. 5 (for loadings see Table 3). *A. (Apodemus) agrarius* and *A. (Karstomys) mystacinus* were clearly separated by Canonical axis 1, while the remaining samples belonging to the subgenera *Sylvaemus* formed a more or less compact cluster in between. Canonical axis 2 segregated *A. agrarius* and *A. mystacinus* from the subgenus *Sylvaemus* within which *A. sylvaticus* formed the outlying cluster (Fig. 4). Canonical axis 3 further differentiated species of *Sylvaemus*. These species showed gradual separation from *A. uralensis* (negative scores) up to *A. flavicollis* (positive scores).

The classification function resulting from CVA analysis of studied samples was then *a posteriori* applied to individuals of *A. peninsulae*. All individuals of *A. peninsulae* were assigned to *Sylvaemus* samples and not to *A. agrarius*, i.e. species representing the same subgenera (*Apodemus*). For the visualisation of individuals of *A. peninsulae* in morphospace see Figs 4 & 5.

DISCUSSION

Among mammal species, there are differences in the timing of growth among various elements of postcranial skeleton, e.g. the growth of the hind foot is completed much earlier than that of long bones, body and tail (Frynta & Žižková, 1992; Melin et al., 2005). This phenomenon may further complicate the interpretation of correlations among studied traits. However, we avoided this potential problem by including only fully grown individuals in our analyses.

Body size itself may play an important role in the adaptive profile of a species, and is sometimes subject to rapid evolutionary change as clearly demonstrated by the phenomenon of island gigantism reported repeatedly in *Apodemus* (Angerbjörn, 1986; Libois & Fons, 1990; Libois et al., 1993; Sarà & Casamento, 1995). Therefore, it is not particularly surprising that the vast majority of variation we found in postcranial skeleton measurements was explained by the first principal component, and can be attributed to size differences. As this paper is focused on examination of the relationship between morphology and ecology, our discussion will focus solely on the shape component of variance, i.e. on differences in relative size of particular bone segments. The evolution of generalised body size will be elaborated elsewhere on the basis of both cranial and postcranial measurements.

Multivariate distances based on size adjusted data revealed that the main pattern of morphometric variation resembled that of molecular phylogeny. Accordingly, the highest degree of morphological differentiation was found among the subgenera *Apodemus*, *Karstomys* and *Sylvaemus*. However, this does not necessarily mean that ecological interpretations of these differences should be excluded (see Poe, 2005). In general, related species are more likely to share similar ecological strategies, and thus the distribution of ecologically relevant characters would often be expected to follow the same phylogenetic pattern. In our case, the subgenus *Karstomys* contains only rock-dwelling species, but *A. agrarius* exhibits a fairly exceptional ecological strategy within the subgenus *Apodemus*. For this reason, we included in our analyses *A. peninsulae*, the other representative of the subgenus *Apodemus* from East Asia, which exhibits ecological requirements similar to those of some European *Sylvaemus* species. Interestingly, cluster analysis placed *A. peninsulae* outside the *Karstomys*-*Sylvaemus* cluster, but not together with *A. agrarius*. In the morphospace of the first two canonical axes, *A. peninsulae* is placed closer to the *Sylvaemus* species, but still in the direction towards *A. agrarius*. This seems to support the intuitive view that the ecology of the species is somewhat associated with the shape of its postcranial skeleton.

There is another procedure that may be used to verify the adaptive nature of observed morphological change, i.e. to evaluate agreement of our results with *a priori* hypotheses concerning the relationships between morphology and locomotor performance (see Introduction). Comparison of the characters responsible for observed morphological variation with ecological parameters of studied species, suggests the following functional interpretations. The first canonical root differentiates studied subgenera according to the degree of their subterranean and digging activity. *A. agrarius* possesses relatively short ears and tail (LAU, LCD), short and robust tibia (LT1, LT2, WT1), stout ilium (WP2) and robust ulna (WU), i.e. the characters likely associated with burrowing (i.e. partly subterranean and fossorial) mode of life of this species (e.g. Heráň, 1962a; 1962b; Hildebrand, 1985; Stein, 2000; Elissamburu & Vizcaíno, 2004; Samuels & Van Valkenburgh, 2008). However, contrary to the functional prediction (see Dobroruka, 1960; Heráň, 1962a; Schich, 1971), *A. agrarius* has relatively long lumbar vertebra (VBL, but see below) and narrow femoral neck (WF3). The opposite is true for *A. mystacinus*, the petricolous, non–burrowing species, for which the long tail (with balance and support function), long tibia, and short lumbar vertebra (Youlatos, 1999) can be of high importance when moving in a rocky environment (vertical jumping). The *Sylvaemus* species occupy an intermediate position along the first canonical root in accordance with their ecological habits (beside burrows they also frequently use ground and above-ground nests) and behaviour (jumping activity, see results of behavioural tests of Frynta (1994) under Introduction), which differ from both *A. agrarius* and *A. mystacinus*. These species form a compact cluster despite supposed variation in the degree of usage of subterranean space among individual species/populations.

The second canonical root separated *Sylvaemus* species (with *A. sylvaticus* in the most extreme position) from *A. agrarius.* This arrangement of studied samples is most likely due to the characters associated with fast terrestrial movement and climbing being opposed by those characters associated with subterranean and digging activity (range of characters, which could not be enforced along Canonical axis 1, recognised as digging – vertical jumping functional sequence; e.g. short LTP1 may be convenient for digging as well as for vertical jumping). The burrowing and digging species - *A. agrarius*, which inhabits

compact vegetation layer hindering fast movement, is characterised by a relatively short ears (LAU), short distal part of hindlimb (LT2, MET, LTP1), short forelimb (LH2, LU1) with long olecranon (OLE) and long (LP2) and broad (WP1) ischiopubis (e.g. HERÁŇ, 1962a; 1962b; HILDEBRAND, 1985; STEIN, 2000; ELISSAMBURU & VIZCAÍNO, 2004; SAMUELS & VAN VALKENBURGH, 2008). While the relatively short post-acetabular part of pelvis (LP2) and long distal elements of the hindlimb (LT2, MET, LTP1) are probably important characters for *Sylvaemus* species inhabiting open microhabitats where fast running or even hopping movement on hindlimbs are used when travelling rapidly (e.g. HERÁŇ, 1962b; SCHICH, 1971). Hopping was reported only in *A. sylvaticus* (DIETERLEN, 1965; NIETHAMMER, 1978) and may be responsible for separation of this species in morphospace along the second canonical axis. Relatively long distal elements of the hindlimb and long forelimbs (with short olecranon) can be further linked with climbing activity (POLK et al., 2000; SAMUELS & VAN VALKENBURGH, 2008; for functional morphology of climbing see also CARTMILL, 1985), which was reported in *A. sylvaticus* and *A. flavicollis*.

The length of lumbar vertebra (VBL) and position of femoral head (WF4) contribute most to the third canonical axis. It differentiates among the *Sylvaemus* species, which are arranged successively in morphospace with *A. uralensis* (probably the least vertically active form of the subgenus *Sylvaemus*) and *A. flavicollis* (frequently performing vertical activity – see Introduction) being in extreme positions. This may be easily interpreted as adaptations: short length of lumbar vertebra found in *A. flavicollis* is possibly associated with vertical leaping (see YOULATOS, 1999 and references therein) and loose femoral head with high degree of lateral movement of the hind limb, the character functionally associated with climbing (DOBRORUKA, 1960).

In conclusion, morphometric examination of postcranial skeleton has revealed considerable variation among subgenera. Interspecific differences usually follow functional predictions associated with ecological habits of species. However, there were found only subtle morphological differences among individual *Sylvaemus* species, in spite of variability of their ecological requirements. This finding may indicate that *Sylvaemus* species possess a majority of generalized morphological features as a result of trade-offs between different habits, which can constrain evolution of special traits on postcranial skeleton (for all species e.g. digging as well as fast running can be of high importance, see also HILDEBRAND, 1985). Ecological diversification of this subgenus can be also explained by body size itself. For proper understanding of the relationship between morphology and ecology, additional comparative data on performance of individual species are urgently required. *Apodemus* species also provide an opportunity to extend our knowledge through additional morphological studies such as e.g. evaluation of character displacement by comparing *Apodemus* populations occurring in sympatry with their counterparts occurring in allopatry (for such a study on cranial measurements see MIKULOVÁ & FRYNTA, 2001).

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Appendix 1 – Origin (localities) and sample size of studied species

Apodemus (*Sylvaemus*) *flavicollis*: Central Europe – 31 specimens from the Czech Republic (Prague). the Middle East – 14 specimens from eastern Turkey (Güzyurdu 1, Kabaca 1), Iran (Gholaman 9) and Armenia (surroundings of Erevan 3).

Apodemus (*Sylvaemus*) *witherbyi*: the Middle East – 49 specimens from eastern Turkey (Seyfe 1, Güzyurdu 4, Yalnizcam Gecidi 1, Bagdasan 4, Aydoglu 1, Damar 1, Kabaca 2, Sirbasan 9) and Iran (Vali Abad 2, Gholaman 7, Yasuj 12, Abshar 2, Sivand 1, Shiraz 2). Note: This species is also referred as *A. hermonensis* Filippucci, Simson, and Nevo, 1989 or *A. iconicus* (Heptner, 1948), see KRYŠTUFEK (2002).

Apodemus (*Sylvaemus*) cf. *hyrcanicus*: the Middle East – 25 specimens from Iran (Asalem 15, Now Kandeh 10). Note: *A. hyrcanicus* was described from the Hyrcanian Reserve in Azerbaijan (VORONTSOV et al., 1992) some 80km north of one of our sites in Asalem. Its conspecificity with our material from Iran is thus probable, but not certain (MACHOLÁN et al., 2001).

Apodemus (*Sylvaemus*) *sylvaticus:* Central Europe – 33 specimens from the Czech Republic (Prague).

Apodemus (*Sylvaemus*) *uralensis*: Central Europe – 23 specimens from the Czech Republic (southern Moravia: Dyjákovičky); the Middle East – 37 specimens from eastern Turkey (Seyfe 10, Güzyurdu 2, Yalnizcam Gecidi 4, Bagdasan 3, Damar 8, Kabaca 8), Armenia (surroundings of Erevan 1) and Azerbaijan (Zakataly Reserve 1).

Apodemus (*Karstomys*) *mystacinus*: the Middle East – 31 specimens from Syria (Quanawat 17, Burqush 1, Slinfeh 12, Sarghaya 1).

Apodemus (*Agrarius*) *agrarius*: Central Europe – 22 specimens from the Czech Republic (Opava 18, Krásná Lípa 4).

Apodemus (*Agrarius*) *penninsulae:* 7 specimens from the Russian Far East (the vicinity of the town Vyazemskiy, district Khabarovsk).

For details of the localities see the following papers: the Middle East - FRYNTA et al. (2001); Prague - MIKULOVÁ & FRYNTA (2001); *Karstomys* - VOHRALÍK et al. (2002).

Appendix 2 – Standard descriptive statistics for 31 postcranial and 4 external measurements (in mm). See Table 1 for measurement abbreviations. CE - Central Europe, ME - the Middle East.

	agrarius		*peninsulae*		*mystacinus*		*witherbyi*		*sylvaticus*		*flavicollis CE*		*flavicollis ME*		*hyrcanicus*		*uralensis ME*		*uralensis CE*	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
LC	94.8	4.27	95.8	8.29	112.2	7.75	98.2	5.61	94.2	4.31	103.4	5.75	100.0	5.67	100.7	7.58	92.7	4.02	89.2	4.00
LCD	76.8	7.06	95.6	14.06	125.0	10.50	101.6	7.28	85.4	5.24	103.9	7.66	104.0	7.81	100.8	6.62	94.7	7.05	81.3	4.08
LTP	18.3	0.90	22.9	1.57	24.6	0.98	21.5	0.90	20.8	0.81	23.5	1.01	22.7	1.30	22.8	0.71	20.9	0.74	18.4	0.73
LA	12.1	0.35	15.3	1.77	18.6	1.69	16.2	0.93	15.7	0.69	17.2	1.05	17.3	1.03	16.6	0.99	14.9	0.72	13.8	1.10
LT	18.41	0.81	22.26	1.29	26.13	1.37	21.72	0.86	20.60	0.91	22.27	0.84	22.42	1.33	22.68	0.86	20.83	0.84	18.84	0.52
LT2	7.29	0.41	8.20	0.32	10.77	0.65	8.52	0.40	8.82	0.53	8.81	0.50	8.84	0.56	8.80	0.41	8.35	0.43	7.36	0.25
WT1	1.75	0.09	2.10	0.24	1.94	0.16	1.66	0.13	1.50	0.14	1.78	0.11	1.87	0.20	1.81	0.17	1.62	0.12	1.36	0.08
WT2	0.94	0.06	1.08	0.08	1.18	0.09	0.98	0.08	0.90	0.08	1.07	0.09	1.08	0.08	1.04	0.09	0.90	0.07	0.82	0.03
LF	16.19	1.14	19.34	1.20	21.79	1.20	18.55	0.93	16.94	0.74	18.75	0.96	19.19	1.32	18.79	1.10	16.81	0.89	15.61	0.53
WF1	2.08	0.19	2.46	0.29	2.72	0.27	2.37	0.23	1.99	0.19	2.27	0.18	2.34	0.18	2.28	0.25	1.95	0.16	1.74	0.09
WF2	1.44	0.11	1.70	0.20	1.90	0.18	1.50	0.14	1.35	0.12	1.57	0.14	1.68	0.16	1.56	0.15	1.40	0.09	1.22	0.08
WF3	0.90	0.06	1.10	0.12	1.33	0.10	1.09	0.09	0.98	0.08	1.14	0.08	1.15	0.07	1.11	0.07	0.98	0.07	0.88	0.04
WF4	0.83	0.12	1.02	0.09	1.08	0.12	0.81	0.14	0.72	0.11	0.90	0.13	0.89	0.10	0.84	0.11	0.69	0.12	0.61	0.08
LP	18.62	1.13	19.86	1.43	22.87	1.56	19.34	1.06	17.21	0.91	19.76	1.29	19.42	1.09	19.32	1.05	17.19	0.94	16.90	0.62
LP2	5.44	0.49	6.21	0.56	7.25	0.75	5.90	0.43	5.08	0.34	6.06	0.42	5.99	0.45	5.85	0.47	5.19	0.37	5.01	0.24
WP1	6.92	0.63	7.38	0.56	8.82	0.76	7.28	0.52	6.51	0.41	7.29	0.70	7.21	0.45	7.12	0.63	6.69	0.47	6.47	0.34
WP2	1.28	0.11	1.50	0.11	1.22	0.09	1.25	0.12	1.26	0.19	1.55	0.16	1.33	0.14	1.36	0.12	1.26	0.13	1.21	0.12
LSF	5.80	0.47	6.01	0.60	7.05	0.61	6.09	0.43	5.17	0.39	6.27	0.61	5.96	0.45	5.95	0.57	5.40	0.43	5.34	0.29
WSF	2.52	0.16	2.43	0.25	3.37	0.27	2.63	0.18	2.10	0.24	2.61	0.21	2.54	0.12	2.71	0.25	2.42	0.19	2.25	0.14
SW1	6.62	0.50	7.37	0.46	8.36	0.78	7.21	0.45	6.88	0.34	7.56	0.54	7.44	0.44	7.36	0.59	6.42	0.43	6.05	0.29
SW2	4.44	0.41	5.05	0.40	5.97	0.56	4.72	0.33	4.56	0.27	4.95	0.37	4.77	0.25	4.78	0.41	4.35	0.34	4.17	0.22
VBW	4.65	0.38	5.70	0.32	6.46	0.55	5.44	0.34	5.03	0.30	5.59	0.37	5.50	0.33	5.50	0.40	4.67	0.36	4.38	0.22
VBL	3.19	0.26	3.35	0.29	3.59	0.30	3.18	0.20	2.88	0.20	3.05	0.19	3.22	0.24	3.20	0.29	3.00	0.23	2.92	0.17
LU	14.28	0.74	16.58	0.78	18.64	0.67	15.88	0.59	15.17	0.53	16.32	0.56	16.64	0.88	16.88	0.67	15.04	0.55	13.80	0.28
OLE	1.95	0.25	2.36	0.19	2.36	0.12	1.99	0.11	1.74	0.13	2.14	0.12	2.07	0.15	2.08	0.12	1.85	0.10	1.71	0.08
WU	1.69	0.10	2.04	0.11	1.98	0.11	1.79	0.14	1.60	0.11	1.92	0.11	1.79	0.09	1.93	0.13	1.69	0.09	1.59	0.08
LH	12.15	0.71	14.00	0.94	15.80	0.80	13.66	0.60	12.63	0.52	13.87	0.67	14.20	0.73	14.25	0.77	12.65	0.54	11.84	0.59
LH1	5.13	0.99	5.84	0.61	6.79	0.46	5.82	0.32	5.11	0.33	5.85	0.45	5.76	0.40	5.92	0.43	5.29	0.36	5.12	0.27
WH1	2.35	0.11	2.97	0.16	2.82	0.22	2.48	0.17	2.22	0.16	2.64	0.18	2.55	0.15	2.56	0.18	2.32	0.10	2.19	0.09
WH2	0.81	0.05	1.16	0.12	1.13	0.09	0.91	0.06	0.79	0.06	0.90	0.07	0.94	0.06	0.92	0.09	0.83	0.04	0.81	0.03
WH3	2.72	0.40	3.38	0.19	3.61	0.14	3.07	0.14	2.63	0.16	3.12	0.15	3.20	0.13	3.23	0.17	2.90	0.20	2.63	0.04
LS	10.25	0.56	11.56	0.50	12.74	0.73	10.95	0.60	10.06	0.38	11.16	0.54	11.29	0.76	10.96	0.56	10.14	0.51	9.66	0.31
WS1	6.91	0.50	7.46	0.53	8.64	0.69	7.69	0.48	6.58	0.46	7.47	0.47	7.50	0.42	7.49	0.61	6.97	0.37	6.51	0.30
WS2	7.57	0.50	7.90	0.72	9.20	0.76	8.36	0.49	7.42	0.49	8.16	0.54	8.48	0.47	8.19	0.63	7.58	0.37	7.21	0.32
MET	7.33	0.38	9.60	0.59	10.02	0.39	8.94	0.33	8.82	0.35	9.58	0.33	9.33	0.41	9.51	0.28	8.90	0.35	7.80	0.31

Appendix 3 - Mahalanobis squared distances computed from data adjusted by Mosimann method (size-free data) .
CE - Central Europe, ME – the Middle East, ag – *A. agrarius*, ur – *A. uralensis*, sy – *A. sylvaticus*, fl – *A. flavicollis*, wi – *A. witherbyi*, hy – *A. hyrcanicus*, pe – *A. peninsulae*, my – *A. mystacinus*

	ag	ur CE	ur ME	my	fl CE	fl ME	sy	wi	hy	pe
ag		75.3	88.0	120.9	81.3	80.1	106.7	77.7	70.4	86.7
ur CE	75.3		14.9	62.5	43.1	38.1	44.2	22.9	27.4	81.5
ur ME	88.0	14.9		51.7	38.1	22.5	36.7	16.8	16.8	75.1
my	120.9	62.5	51.7		68.4	49.1	81.6	37.4	52.4	109.9
fl CE	81.3	43.1	38.1	68.4		22.5	37.0	26.2	20.8	74.4
fl ME	80.1	38.1	22.5	49.1	22.5		30.0	14.6	9.6	72.1
sy	106.7	44.2	36.7	81.6	37.0	30.0		36.6	30.6	115.6
wi	77.7	22.9	16.8	37.4	26.2	14.6	36.6		13.1	74.6
hy	70.4	27.4	16.8	52.4	20.8	9.6	30.6	13.1		64.7
pe	86.7	81.5	75.1	109.9	74.4	72.1	115.6	74.6	64.7	

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Halofenozide affects sexual behaviour, cuticular hydrocarbons and reproduction in the female German cockroach *Blattella germanica* (Dictyoptera, Blattellidae)

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ABSTRACT. Halofenozide, a dibenzoylhydrazine insect growth regulator, was applied topically to female individuals of the most prevalent German cockroach species, *Blattella germanica*, and its effects on sexual behaviour, cuticular hydrocarbons and reproduction were investigated. Dissection of treated females showed clearly reduced numbers of oocytes and volume of basal oocytes. Interestingly, the ecdysteroid amounts were also significantly lower. Characterization of the cuticular hydrocarbons by gas chromatography showed 13 major compounds, including the female contact sex pheromone precursor. It was clear that halofenozide application resulted in significantly lower amounts of all the investigated cuticular components. Finally, behavioural tests revealed that halofenozide treatment of females caused a significant decrease in sexual receptivity of the untreated conspecific males. Thus, exposure to the ecdysteroid agonist can negatively affect the male's response to a calling female, which is linked with and most likely caused by lesser production of female contact sex pheromone and a delay in the up-regulation of ecdysteroid amounts, and in turn this provoked an obvious lower oocyte number and basal oocyte size as measures of reproduction.

KEY WORDS : *Blattella germanica*, Halofenozide, Reproduction, Sexual behaviour, Cuticular hydrocarbons, Ecdysteroids.

INTRODUCTION

In insects, the steroid moulting hormone, 20-hydroxyecdysone (20E), and the sesquiterpenoid, juvenile hormone (JH), play a central role in the regulation of growth, development and reproductive processes, and are considered as potential specific target sites for pest control (DHADIALLA et al., 1998; GÄDE & HOFFMANN, 2005). Due to secondary effects of conventional insecticides on the environment, a class of selective insect growth regulators (IGRs) that mimic or antagonize the action of 20E and JH, has been developed. While success in the discovery of JH mimetics came much earlier, it is only in the last decade that insecticides that act as agonists of 20E, have been discovered (DHADIALLA et al., 2005).

RH-5849 (1,2-dibenzoyl-1-tert-butylhydrazine), tebufenozide (RH-5992) and methoxyfenozide (RH-2485) are the first members of this new class of compounds, namely dibenzoylhydrazines, that induce a precocious and incomplete moulting in several insect orders, especially Lepidoptera (DHADIALLA et al., 1998; 2005). These compounds manifest their biological activity *via* interaction with the ecdysteroid receptor complex in a competitive manner with ecdysteroids and interfere with expression of some genes involved in cuticle synthesis and secretion. Halofenozide (RH-0345) is a novel member of this class of IGRs. Over the last decades, researchers from different laboratories reported the effects of several of these IGRs on the reproductive performance of insects of different orders, especially Lepidoptera and Coleoptera (SMAGGHE & DEGHEELE, 1994; DHADIALLA et al., 1998; HOELSCHER & BARRETT, 2003; TAIBI et al., 2003; AMRANI et al., 2004). Interestingly, negative effects were also reported in females and males of Lepidoptera (SMAGGHE et al., 2004). In addition, recent reports speculated on the behavioural effects induced by ecdysteroid agonists. In this way, HOELSCHER & BARRETT (2003) showed that the male moth's ability to respond to a calling female is negatively affected by treatment with methoxyfenozide, inhibiting the male's locomotory activity, and this, in turn, caused an obvious reduction in reproduction. BARRETT (2008) also demonstrated that exposure of adult moths of the codling moth, *Cydia pomonella*, to methoxyfenozide-treated surfaces resulted in a negative impact on male responsiveness to calling females and synthetic pheromone lures.

The chemistry, biochemistry and behavioural ecology of the sexual communication system of German cockroaches are relatively well understood (GEMENO & SCHAL, 2004). Elaborate mating display is controlled by various volatile and non-volatile (sex) pheromones, but the mechanisms affecting the regulation of these chemicals are still not fully understood due to a lack of sufficient physiological data. SCHAL & SMITH (1990) reviewed the potential role of brain, *corpora allata* and various hormones such as ecdysteroids, in pheromone production in various cockroach species, with most detailed studies in *Blattella germanica* and *Supella longipalpa*. In *B. germanica*, a number of cuticular hydrocarbons serve as biosynthetic precursors to specific contact sex pheromones

that are perceived by antennal sensillae and function as contact primers of sex pheromone triggering behavioural phase transition (BLOMQUIST et al., 2005).

The most prevalent German cockroach *B. germanica* is well known for its high economic and medical importance and its strong resistance to conventional insecticides (SCOTT et al., 1990). Thus new target insecticides are urgently needed to reduce resistance pressure. In this study, adult females of *B. germanica* were tested to evaluate the activity of the ecdysteroid agonist halofenozide on their reproduction. With these experiments we aimed to confirm the ecdysteroid agonistic effects of halofenozide and also to try to understand the action of those molecules on the reproductive process and insect behaviour. This component was firstly tested on the female for its potential activity on ovaries, total ecdysteroid amounts and cuticular hydrocarbon profiles. The observed perturbation of adult sexual behaviour is discussed.

MATERIALS AND METHODS

Insect rearing

Colonies of *B. germanica* were reared in plastic boxes with dog food pellets and water *ad libitum*. They were kept at 27±1°C under a 12h light:12h dark regime and 70% relative humidity (HABES et al., 2006). None of the insects had been in previous contact with any insecticides.

Insecticide and treatment

Halofenozide of technical grade (>95% pure; Rohm and Haas Co., Spring House, PA, USA) was topically applied after dilution in acetone (10µg in 3µl per insect) to newly emerged adult females. Control insects were treated with acetone alone. All the insects were kept under the same conditions as given above. Experiment procedures for each test are outlined below.

Ovarian parameter

Halofenozide was administrated topically to newly emerged females (less than 3 hours after adult emergence). Each treated female was immediately paired with one untreated male in a plastic box (9.5cm x 6.5cm x 2cm) containing food and water. Adult females from control and treated series were sampled at 0, 2, 4 and 6 days during the adult life and their ovaries dissected out. After removal of circumovarian fat body, the numbers of oocytes per pair of ovaries were recorded and the volume of the basal oocyte was determined as described in LAMBREAS et al. (1991). Six to ten replications were done for each series (AMRANI et al., 2004).

Ecdysteroid extraction and quantification

Six days after treatment, ecdysteroids were extracted from individual whole adult female bodies with methanol (2ml) by sonication (2-3min) as previously described (BERGHICHE et al., 2008). Samples were centrifuged at 5,000g for 10min, and the supernatants evaporated. Each extract was resuspended in 500µl of phosphate buffer (0.1M; pH7.4) and analyzed in an enzyme immunoassay (EIA) using a rabbit polyclonal B antibody against 20E coupled to peroxidase as an enzymatic tracer and tetramethyl benzidine as a color reagent. Each experiment was replicated 6 times according to previous publications (AMRANI et al., 2004; ARIBI et al., 2006). Data are expressed in pg 20E equivalents per insect. Antibodies and tracer were kindly supplied by Dr. J.-P. Delbecque (Laboratoire de Neuroendocrinologie, Université de Bordeaux I, France).

Gas chromatography analyses of cuticular hydrocarbons

After 1, 3 and 6 days, insects were frozen for about 5min at -20°C and extracted individually for 5min at room temperature in 1ml of distilled pentane containing 10µg *n*-octadecane (C18) as internal standard (IS). The extracts were stored at -20°C until analysis. Extracts were then concentrated to about 100µl under a nitrogen flow, and aliquots of 1µl analyzed by gas chromatography (GC) using a CP-9000 of Chrompack (Bergen op Zoom, The Netherlands) fitted with a split-splitless injector (30s split, 25ml/min) and a flame-ionisation detector. Injector and detector temperatures were 260°C and 280°C, respectively. The analytical column used was an apolar CP-Sil 5 fused silica capillary column (25m x 0.25mm ID, Chrompack). Helium was used as carrier gas at a velocity of 35cm/s at 120°C. The oven temperature was programmed from 140°C to 280°C at 3°C/min. The signal was recorded and integrated on a computer fitted using Maestro software (Chrompack). Ten repetitions were done for each series. Estimation of the proportion obtained was done with a normalized area quantitative method (EVERAERTS et al., 1997). The quantification (ng per insect) of each component was calculated using the response factor of the internal standard.

Sexual behaviour assay

All the observations were made under a dim red light in plastic boxes (19cm x 13cm x 4cm) during the first hours of the scotophase, which correspond to the maximal period of sexual activity in this species (LIANG & SCHAL, 1993). From the continuous colony, 80 virgin adults (newly ecdysed adults, 40 males and 40 females) were randomly selected and kept separated. In this species, the females are responsible for the male sexual attraction; so, in the treated series, only the females received halofenozide. For the experiments we used insects 6 days into the adult stage when females and males are sexually mature (SCHAL et al., 1997; LIHOREAU et al., 2008). Behaviour observations were made on 20 separate couples. Each insect pair was introduced into an observation box without any anesthesia because CO_2 is known to affect insect behaviour (GUERENSTEIN & HILDEBRAND, 2008; CHAMPION DE CRESPIGNY & WEDELL, 2008). The number of male wing raisings, a characteristic response when a mature virgin female is encountered (LIHOREAU et al., 2008), was determined during a 15min period (KIM et al., 2004; LIHOREAU et al., 2008).

Statistical analysis

Results are presented as the mean ± SEM. Significance of differences between means was estimated by a Stu-

dent's *t*-tests at $p<0.05$. A multivariate analysis (MANOVA) was conducted on relative amounts of the cuticular components in relation to treatment and duration of exposure (KIM et al., 2004). Statistical analyses were performed using MINITAB 12.21 software (Minitab Inc., State College, PA, USA).

RESULTS

Ovarian measurements

As shown in Fig. 1, the number of oocytes per paired ovaries in female controls increased during sexual maturation (0–2 days-old; $p<0.001$), and decreased at days 4 and 6 ($p<0.001$) (beginning of ovulation). Treatment with halofenozide on newly emerged females of *B. germanica* significantly reduced the number of oocytes present at days 2 ($p=0.002$) and 4 ($p=0.016$) compared to controls of the same age. At day 6, the absence of differences between controls and the treated series is probably due to the reduction of oocyte numbers following egg–laying in controls and stable values in the treated series.

Fig. 2 illustrates that the volume of the basal oocyte in controls increased during sexual maturation from 0.0040±0.0003 mm^3 to 0.090±0.004 mm^3, while halofenozide–treated females had significantly smaller volumes at days 2, 4 ($p=0.006$) and 6 ($p<0.001$) (Fig. 2).

Ecdysteroid amounts

During the first 6 days, ecdysteroid amounts in control females reached 7.34±0.57 pg/insect. Halofenozide-treated females (10µg) had about 26% lower ecdysteroid titers (5.46±0.54pg/insect) ($p=0.039$).

Effects on cuticular hydrocarbon profiles

In *B. germanica*, the chemical nature of the cuticular hydrocarbons has previously been described (RIVAULT et al., 2002). In our GC analyses, 25 peaks was detected according to RIVAULT et al. (2002) but, because of low concentrations of some compounds, we chose the 13 major ones that were present at concentrations of >500ng/insect in all the investigated samples (*i.e.*, at 6 day, peak 1: 602±72ng, peak 12: 8334±999ng) (Fig. 3). As described by RIVAULT et al. (2002), the compounds were identified as *n*-heptacosane (peak 1), 9-, 11- and 13-methylheptacosane (peak 2), 5-methylheptacosane (peak 3), 3-methylheptacosane (peak 4), *n*-nonacosane (peak 5), 9-, 11-, 13- and 15-methylnonacosane (peak 6), 7-methylnonacosane (peak 7), 5-methylnonacosane (peak 8), 11,15- and 13,17-dimethylnonacosane (peak 9), 3-methylnonacosane (peak 10), 5,9- and 5,11-dimethylnonacosane (peak 11), 3,7-; 3,9- and 3, 11-dimethylnonacosane (peak 12) and 11-, 13- and 15-methyltriacontane (peak 13). Peak 12 corresponds to the most abundant component of the female contact sex pheromone precursor (CHASE et al., 1992). All the obtained chemical profiles are comparable. Quantitative differences between the control and treated groups at days 1, 3 and 6 were observed (Fig. 4). The concentration of each component did not depend on the length of the treatment: *i.e.*, peak 12: day 1 (1316±175ng), day 3 (1478±286ng), day 6 (1837±300ng). A multivariate analysis revealed that the amounts of all the investigated peaks were significantly lower (MANOVA: $p<0.001$) in the insects treated with halofenozide than in the control insects.

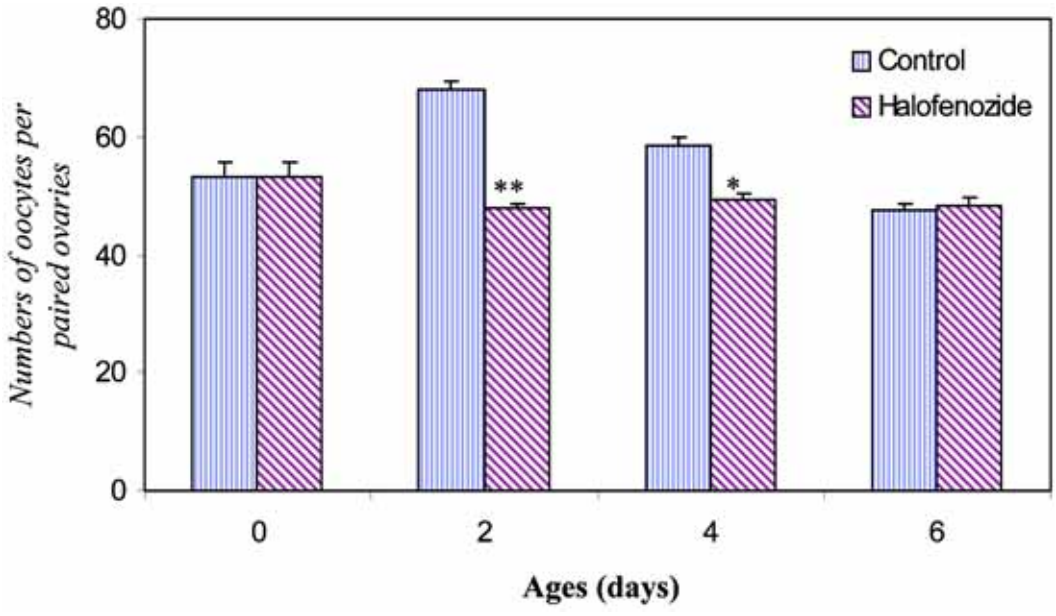

Fig. 1. – Effect of topical application of halofenozide (at day 0) on newly ecdysed female adults of *B. germanica* on the numbers of oocytes per paired ovaries. Value is the mean ± SEM (n=6-10). Asterisks indicate a significant difference between control and treated series of the same age (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

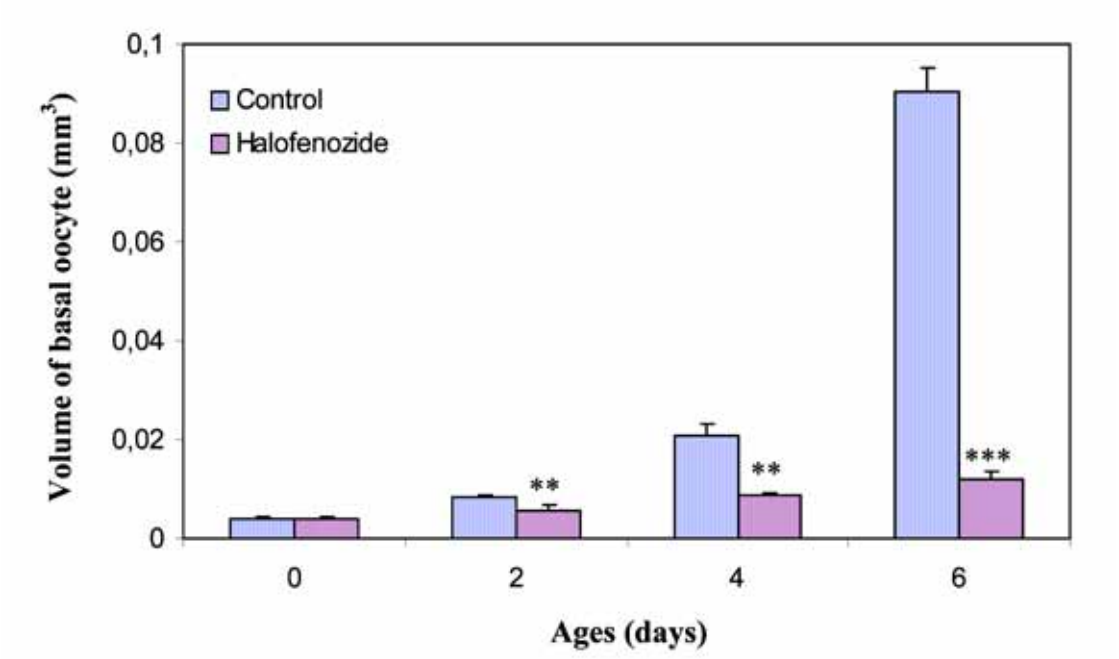

Fig. 2. – Effect of topical application of halofenozide (at day 0) to newly ecdysed female adults of *B. germanica* on the volume of the basal oocyte. Value is the mean ± SEM (n=6-10). Asterisks indicate a significant difference between control and treated series of the same age (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

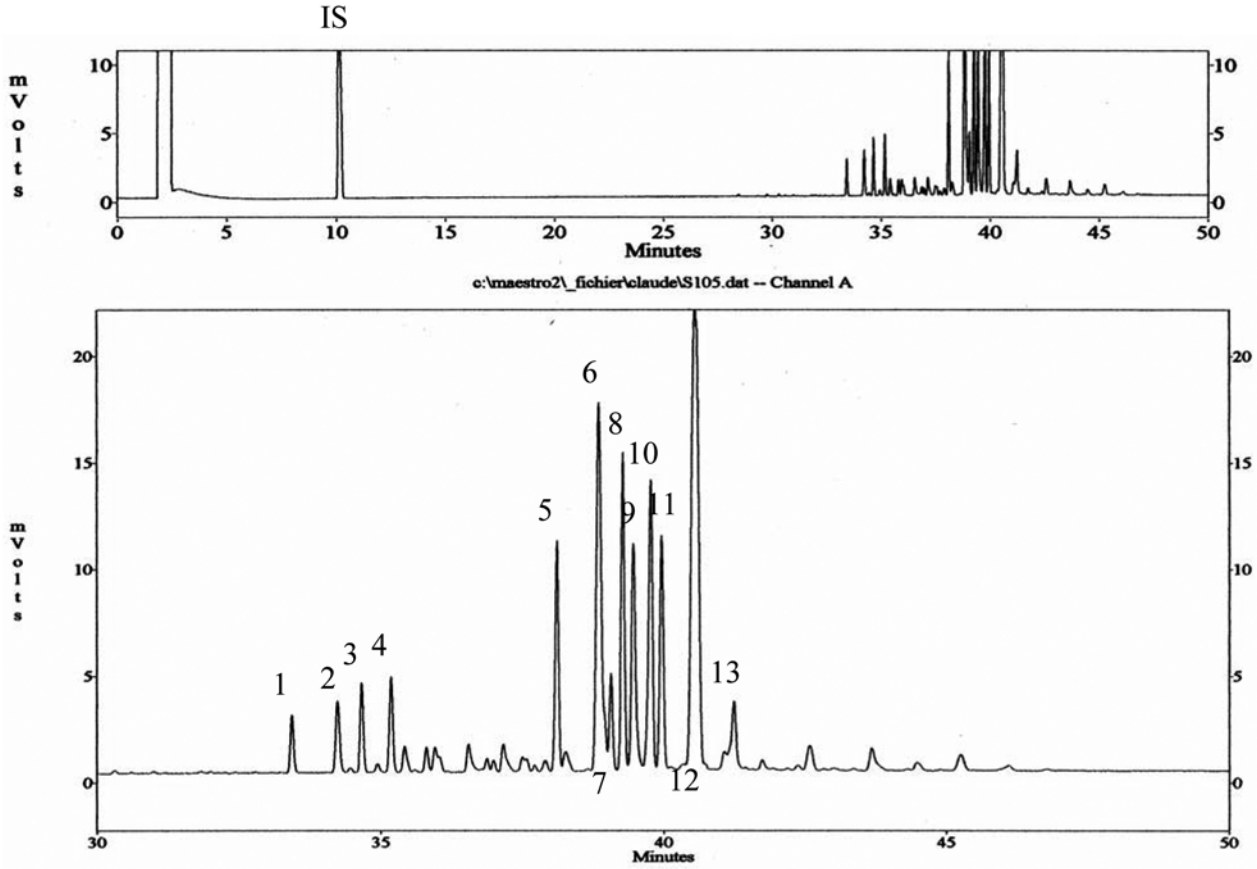

Fig. 3. – Typical gas chromatography profile of an extract of cuticular hydrocarbons of *B. germanica* adults of both sexes. Peaks 1 to 13, see results; IS, internal standard.

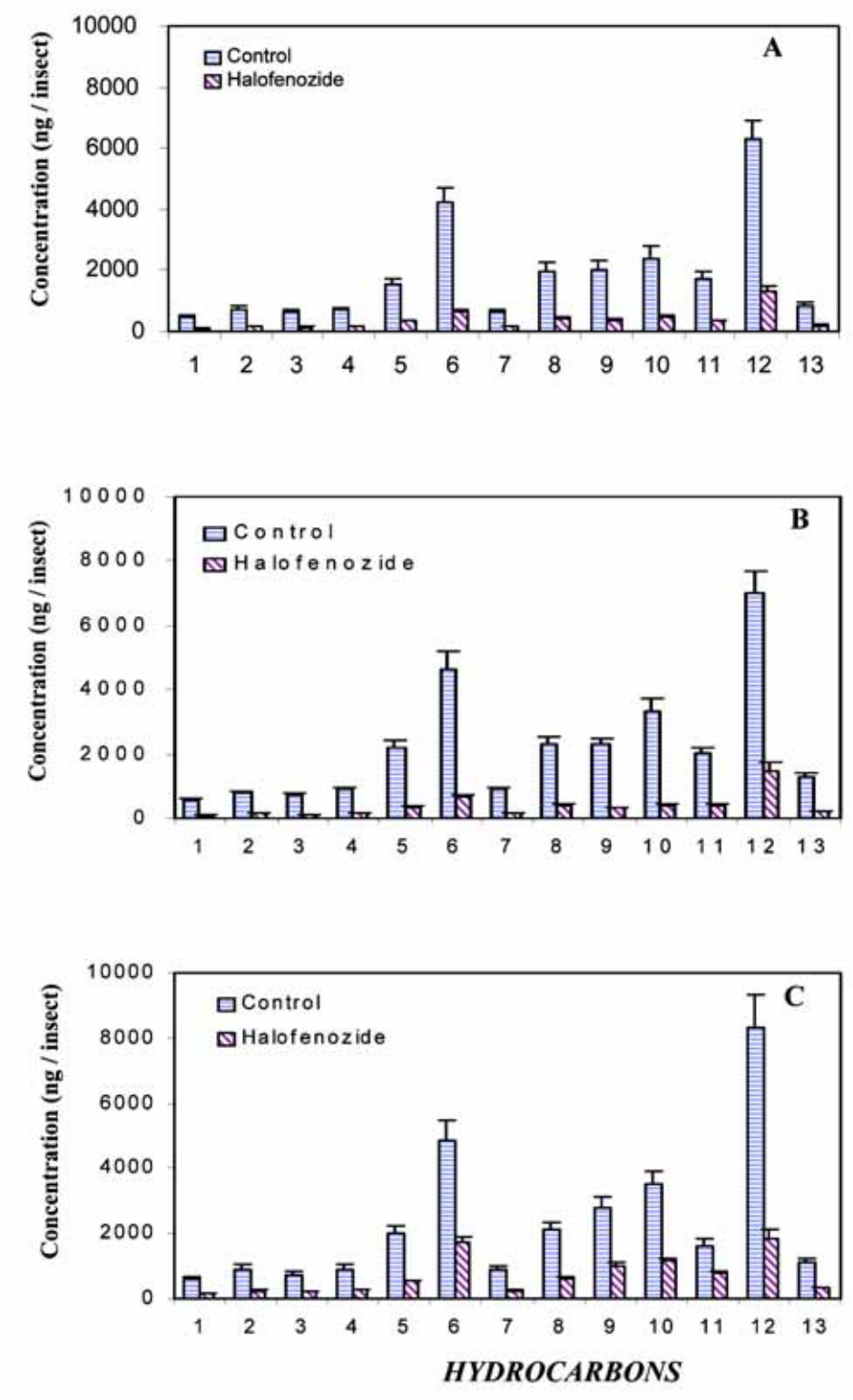

Fig. 4. – Quantification (ng/insect) of the 13 investigated major cuticular hydrocarbons in control and treated females of *B. germanica* of various ages (mean ± SEM, n=10). (A: day 1, B: day 3, C: day 6).

Sexual behaviour

To test the potential "sex-appeal" of a female, we chose to measure the number of male wing-raisings, which are generally correlated with the sexual maturity of the female (LIHOREAU et al., 2008). Wing-raising is a characteristic posture of the male that occurs only after contact with a virgin female. This behaviour does not imply that the female mounts the male to copulate, but it is a good indication of the sexual "excitation" of the male.

We observed (data not shown) that the number of male antennae contacts with a female did not differ significantly between control and treated series. Thus, antennae contact between the sexes is not related to the male wing-raising effect. The 6-day-old males in the controls showed 14.3±1.3 wing-raisings in response to a sexually mature female. It was striking that when halofenozide was applied to the newly emerged females, significantly ($p=0.004$) fewer male wing-raisings (9.1±1.0) were noted.

DISCUSSION

Development and reproduction processes in insects are orchestrated by ecdysteroids, JHs and neuropeptides (GÄDE & HOFFMANN, 2005) and it is clear that any exogenous sources of hormones, synthetic agonists or antagonists that interfere with the homeostasis of the insect hormones can be exploited as novel insecticide targets to disrupt normal development and reproduction of pest insects.

In the present study we proved that treatment of newly ecdysed females of *B. germanica* with the ecdysteroid agonist halofenozide resulted in reduced numbers of oocytes and smaller volume of the basal oocyte during the adult life as compared to controls. It is well known that non-steroidal ecdysteroid agonists cause a decline in reproduction in different insect orders such as a Lepidoptera, Diptera, Coleoptera and Orthoptera (SMAGGHE & DEGHEELE, 1994; DHADIALLA et al., 1998; SOLTANI et al., 1998; SUN et al., 2003; TAIBI et al., 2003; AMRANI et al., 2004). Previous studies using various ecdysteroid agonists have shown that these compounds affect ovarian growth in lepidopteran and coleopteran species (FARINOS et al., 1999; SUN et al., 2003; SMAGGHE et al., 2004). Halofenozide was found to reduce both growth and development of oocytes *in vitro* and the thickness of follicular epithelium *in vivo* in mealworms, suggesting a reduction in the synthetic activity of the follicle by structural alterations and/or biochemical modifications (SOLTANI et al., 1998). In *B. germanica*, as in all cockroaches studied to date, vitellogenesis and cyclic maturation of oocytes depend upon JH III synthesis by the *corpora allata* and a decline in JH synthesis occurs just before ovulation (SCHAL et al., 1997). Consequently, the reduced number and volume of oocytes we observed a few days after halofenozide treatment may be due to interference by this hormone agonist with JH or other neuropeptides that regulate ovulation and reproduction, which can subsequently affect the sex communication system. However, the exact mechanism underlying this negative activity of halofenozide remains unknown so far.

For the prototype compound of halofenozide, RH-5849, it was shown that ecdysteroid titers in treated tobacco hornworm *Manduca sexta* larvae were depressed within 4hr after injection (WING et al., 1988); this implies that ecdysteroids and agonists may exert a negative feedback inhibition on hormone biosynthesis in *Pieris brassicae* (BEYDON & LAFONT, 1983) and *Bombyx mori* (GU et al., 2008). ROMANA et al. (1995) found ecdysteroid titers in the haemolymph and ovary to be relatively low in freshly ecdysed females of *B. germanica*, increasing progressively during vitellogenesis, peaking at choriongenesis, and decreasing after ovulation. Our data from EIA measurements showed clearly that halofenozide caused inhibition of the ecdysteroid titers in 6-day-old adult females of *B. germanica*.

In the current experiments, halofenozide was also found to reduce quantitatively the various major cuticular hydrocarbons of female adults of *B. germanica*. In this species, the cuticular hydrocarbons are synthesized at the level of the oenocytes in the abdominal integument (FAN et al., 2003). In insects, cuticular hydrocarbons are synthesized through elongation of fatty acyl-CoA, fatty acid reduction to aldehydes, and fatty aldehyde conversion to alkanes that contain one carbon less (NELSON & BLOMQUIST, 1995). The aldehyde is decarboxylated to hydrocarbon and carbon dioxide by cytochrome P450 enzyme, which requires NADPH and molecular oxygen (REED et al., 1995). In *B. germanica*, the cuticular hydrocarbons consist of a complex mixture of apolar compounds that contain *n*-alkanes, monomethylalkanes and dimethylalkanes (RIVAULT et al., 2002). In addition, six female sex-specific components were identified (CHASE et al., 1992). The most abundant component of the female contact sex pheromone, 3,11-dimethyl-2-nonacosanone (NISHIDA et al., 1974), is derived through hydroxylation and subsequent oxidation of the abundant cuticular hydrocarbon 3,11-dimethylnonacosane (CHASE et al., 1992). Three components derived from this C29-dimethyl ketone with either a methyl, alcohol, or an aldehyde functionality at the C29 position, are also present. Other components with a C27 skeleton, 3,11-dimethylheptacosanone and its alcohol or aldehyde forms also serve as sex pheromones (JURENKA et al., 1989; MORI, 2008), but those components are less abundant on the female cuticular surface. The behavioural activity of 3,11-dimethylheptacosanone is significantly lower than that of its C29 homologues (ELIYAHU et al., 2004). As shown in our data, the compound represented by peak 12, one of the major precursor of the female sex pheromone components, appeared in higher concentration in 6-day-old treated females. Due to its poor GC response, the high molecular weight contact pheromone, 3,11-dimethyl-2-nonacosanone, which appears in very minute quantities in the female, was not detected in our GC traces. This compound is one of the female components that is responsible for the wing-raising posture of the male. Consequently, the accumulation of peak 12 onto the cuticle of the female may certainly be in close connection with a perturbation of the biosynthesis pathway of the specific female hydrocarbons. Regarding our results, we can hypothesize that the reduced amounts of hydrocarbons observed in halofenozide-treated females might have been caused at different levels of synthesis and at delivery and/or trans-

port of these compounds to the surface of the cuticle. The decrease in cuticular compounds might have a secondary influence on sex recognition in the reproductive process and explain the perturbation noted in the male behaviour.

In cockroaches, pheromone production is coordinated with the gonadotropic cycle and the major gonadotropic hormone (*i.e*., JH) has been recruited to act on several target tissues (BLOMQUIST et al., 2005). Previous studies demonstrated that precocene inhibited pheromone production in this family (CHASE et al., 1992; SCHAL et al., 1994), whereas hydroprene, a JH analogue, increased the female pheromone production (SCHAL, 1988). So, as described in various insect species (SLEDGE et al., 2004; LOMMELEN et al., 2006), we can suggest that the quantitative variation observed in the female *B. germanica* cuticular hydrocarbons after halofenozide treatment could be related to reproduction and/or to other unknown processes; this will be due to the synergistic or inhibitory interactions between 20E, JH and other neurohormones (BELLÈS, 1995). In this way, it has been speculated that 20E appears to regulate fatty acyl-CoA elongase. However, little is known of either the enzymology or the molecular biology of hydrocarbon production (BLOMQUIST et al., 2005). BLOMQUIST et al. (2005) noted that 20E and JH induce and repress the synthesis of specific enzymes at the transcription level of pheromone production. In the female housefly, *Musca domestica,* and possibly in other species of Diptera, it appears that during hydrocarbon sex pheromone biosynthesis, ovarian-produced ecdysteroids regulate the synthesis by affecting the activities of one or more fatty acyl-CoA elongation enzyme(s). In Lepidoptera, sex pheromone biosynthesis is often mediated by the pheromone biosynthesis activating neuropeptide (PBAN) through alteration of enzyme activities at one or more steps prior to, or during fatty acid synthesis or modification of the carbonyl group (TILLMAN et al., 1999). So the decrease of hydrocarbon amounts observed in our experiments after halofenozide treatment could be explained by the inhibition of fatty acyl-CoA elongase.

In the German cockroach, sexually receptive females attract males from a distance with a volatile pheromone, namely blattellaquinone, which is emitted at the level of the pygidium (ABED et al., 1993; NOJIMA et al., 2005). When the male is in contact with a virgin female, he recognizes her by his antennae. The presence of a contact sex pheromone (3,11-dimethyl-2-nonacosanone) on the cuticular surface of the female induces the characteristic male wing-raising behaviour (ELIYAHU et al., 2004). This typical behaviour triggers specialized male tergal glands that induce the female to climb onto the back of the male, and so placing her in a precopulatory position (GEMENO & SCHAL, 2004).

In the present study, we showed that when applied on females, halofenozide disturbed the male wing-raisings. The observed decline of this male posture could result in a lower production or lower perception of the contact female sex pheromone due to the lower amount of cuticular compounds. It is known that females of *B. germanica* can already mate 2 days after emergence, but the maximum copulation was observed at day 13 (ABED et al., 1993). In our experiments, no copulation was noted in control or treated series within the first 6 days and the possibility that halofenozide can interfere at the behavioural level, through control of pheromone production or emission during calling, remains realistic.

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Age and size at metamorphosis of half-sib larvae of *Salamandra infraimmaculata* born in the laboratory and raised singly under three different food regimes

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ABSTRACT. *Salamandra infraimmaculata* is an endangered amphibian urodele species inhabiting an unpredictable xeric environment at the edge of its zoogeographic distribution on Mt. Carmel, Israel. This area is characterized by early October rains of short duration forming rock pools where this salamander breeds. Since these shallow ponds dry out rather soon, the larvae have limited time to develop and metamorphose. It is of great importance that they reach this stage of development quickly and attain the largest size possible.

This study examined metamorphosis under experimental conditions using half-sib larvae of the same age raised singly under different food regimes without any stress of density.

It was found that age, mass and length at metamorphosis regressed significantly with food regimes when salamander larvae were raised singly. Thus, a significant difference in age and mass at metamorphosis was found between larvae raised singly on three food regimes. Age was least, and both mass and length were greatest when larvae were fed '*ad-libitum'*.

Minimal and maximal age at metamorphosis increased significantly and mass decreased significantly as food became scarcer.

Consequently, metamorphosis appears to be affected by food resources when density was not a factor. The significance of this finding is discussed.

KEY WORDS : *Salamandra,* Caudata*,* Larval cohorts, Resource effects, Metamorphosis

INTRODUCTION

Several risks are involved in the successful completion of metamorphosis in nature for individuals of the species *Salamandra infraimmaculata* Martens, 1855:

1. Will rain start early and in sufficient quantities to fill the ponds, with sufficient water to sustain larval growth until completion of metamorphosis? The break in rain should be short so that the ponds will not dry out, allowing larvae born early to survive (WARBURG, 1992).

2. Will there be enough food to enable growth (COHEN et al., 2006) of early-born larvae? Will it be enough so that late-born larval cohorts, that may provide food for early-born cannibal larvae (COHEN et al., 2005); will also be able to survive?

3. Will larval density be low enough to enable a short development period and a large size at metamorphois?

The successful survival of post-metamorphs depends on the survival of the larvae in spite of all these risks.

Both age at metamorphosis (measured from their birth in the laboratory), as well as the duration of the larval period (i.e. the time it takes to metamorphose), are of great significance. Moreover, the size attained by the larvae until metamorphosing is of great consequence to the survival of the post-metamorphs. These questions have been addressed in several previous studies on urodeles (WILBUR & COLLINS, 1973). However, the two main factors important to metamorphosis, density and resources (i.e. food), have rarely been analyzed separately except by LICHT (1992) who raised solitary larvae of *Ambystoma gracile,* and both OHDACHI (1994) and KOHMATSU et al. (2001) who raised solitary larvae of *Hynobius retardatus)* to metamorphosis.

In the present study an attempt is made to study the effect of food resources when half-sib larvae were raised singly from birth, with no other larvae competing or preying thus ruling out the density effect. The effect of density on metamorphosis when food is available *ad-libitum*, will be examined separately.

MATERIALS AND METHODS

The study is based on long-term (1974-1998) observations on a single breeding population of *Salamandra infraimmaculata* (WARBURG, 2006; 2007a,). This salamander is a rare and endangered species found in the northern part of Mt.Carmel, Israel.

Gravid females were collected near the breeding ponds and allowed to release their larvae in the laboratory. This salamander species is ovoviviparous, laying eggs containing completely formed larvae wrapped in an egg envelope. The larvae hatch immediately upon contact with water (WARBURG et al., 1978/79). The females were then released back to nature (WARBURG, 2007 b; 2008). During the study period a total of 74 cohorts of half-sib larvae were born in the laboratory. Thus their mothers and the larvae's dates of birth are both known. These cohorts contained 4085 larvae all born to these freshly-collected females. Most of the larvae were released back to the ponds where their mothers were collected, when one day old (see WARBURG, 2009a). Of the remaining larvae, 396 were raised in the laboratory (at room temperature) until

they metamorphosed (COHEN et al., 2005; 2006), while others were raised for an additional 2-5 years after metamorphosis to be released as juveniles back to nature (in preparation).

The larvae were placed singly into 'finger bowls' (13.5cm diameter, 42.4cm^2/larva). Since salamander larvae usually stay on the bottom most of the time, surfacing only to gulp air towards the end of the metamorphic cycle, the calculation is per area rather than per volume. The finger bowls were filled with aged tap water 2cm in depth that was changed daily after feeding.

Larvae received 0.02g minced liver (wet weight) per larva, or 0.01g live *Tubifex* larvae per larva (plenty of food), 0.005g liver per larva or 0.004g live *Tubifex* larvae per larva (little food) and the last group received an unlimited amount of live *Tubifex* larvae during the experiment ('*ad-libitum'*). No significant differences have been found in the effects of these two food items on either size or time to metamorphosis (COHEN et al., 2005). At the age of 18 days the amount of *Tubifex* provided was quintupled and at the age of 23 days the amount of chopped liver was doubled. For 'little' and 'plenty' food conditions, larvae were fed every 3-4 days and the bowls were cleaned of food leftovers 1-1.5hrs after feeding, and filled with fresh water. For '*ad-libitum'* conditions live *Tubifex* larvae were added after cleaning and were always available.

Towards the end of their larval period soil was added enabling the larvae to crawl out and metamorphose.

The data were analysed in several different ways.

1. The means and the standard deviation of each data series were calculated. The latter provided a method by which the variability within the data can be evaluated. It is well known that comparing means is not sufficient since the same mean can be obtained with different data series. The difference between the means of age, mass and length at metamorphosis under three different food regimes was examined by t-tests between the means.

2. In each data series minimum and maximum values as well as the range between them were used as different parameters. These values are to a certain extent related but not necessarily so. Thus, the same range between minimum and maximum can be observed in different data series with different minimum and maximum values. In other words range alone is not sufficient to evaluate data.

3. To compare between two data series I have used regression analyses. Thus, the relationships between age, mass and length were tested using regression analysis.

RESULTS

Age and mass at metamorphosis were studied in a number of half-sib larval cohorts (Fig. 1). The difference in the average age and mass between the different cohorts was not significant. Age, mass and length at metamorphosis regressed significantly with food regimes (R^2=0.9188, R^2=0.9942 and R^2=0.9442 linearly) (Fig. 2 A-C). Likewise, there was a marked difference in both age and length at metamorphosis when larvae were raised on unlimited food (Fig. 2 A; C). Both mass and length increased significantly with age when larvae were raised on 'little' food (R^2=0.829 linear, and R^2=0.824 linear, respectively) but no significant relationship was noticeable when they were fed 'plenty' or *'ad-libitum'*. There was a significant difference (p=0.017) in mass between larvae raised with 'plenty' of food or '*ad-libitum'*, and the difference in length from receiving '*ad-libitum'* or 'little' food, was highly significant (p<0.0001).

The relationship between age, mass and length is shown for individual metamorphs in Fig. 3. The variability among individuals is remarkable. Likewise, the number of larvae metamorphosing varied greatly (Fig. 4). Both inter- and intra-cohort differences can be seen.

Both minimal and maximal age at metamorphosis regressed significantly (Fig. 5 A). Age at metamorphosis increased as food became scarcer (R^2=0.9734 logarithmic, and R^2=0.9235 logarithmic, respectively). Similarly, both minimal and maximal mass at metamorphosis regressed significantly with food regimes (Fig. 5 B). Thus, metamorphs became smaller with food becoming scarcer (minimal mass R^2=0.9981 linear, and maximal mass R^2=0.9169 logarithmic). There was a significant difference in mass among the three food regimes. On the other hand, metamorphs were not significantly shorter when food became scarce (Fig. 5 C).

The range between minimal and maximal age, mass and length decreased significantly when food became scarce (Fig. 6 A-C). Thus, range in age decreased significantly when food became scarce (R^2=0.9959 linear, Fig. 6 A), likewise in mass (R^2=0.9346), and in length (R^2=0.7797) both logarithmic, Fig. 6 B; C).

The differences in age at metamorphosis (60%) between larvae raised on 'little' food or *ad-libitum* exceeded the differences in mass (34%) and length (43.7%).

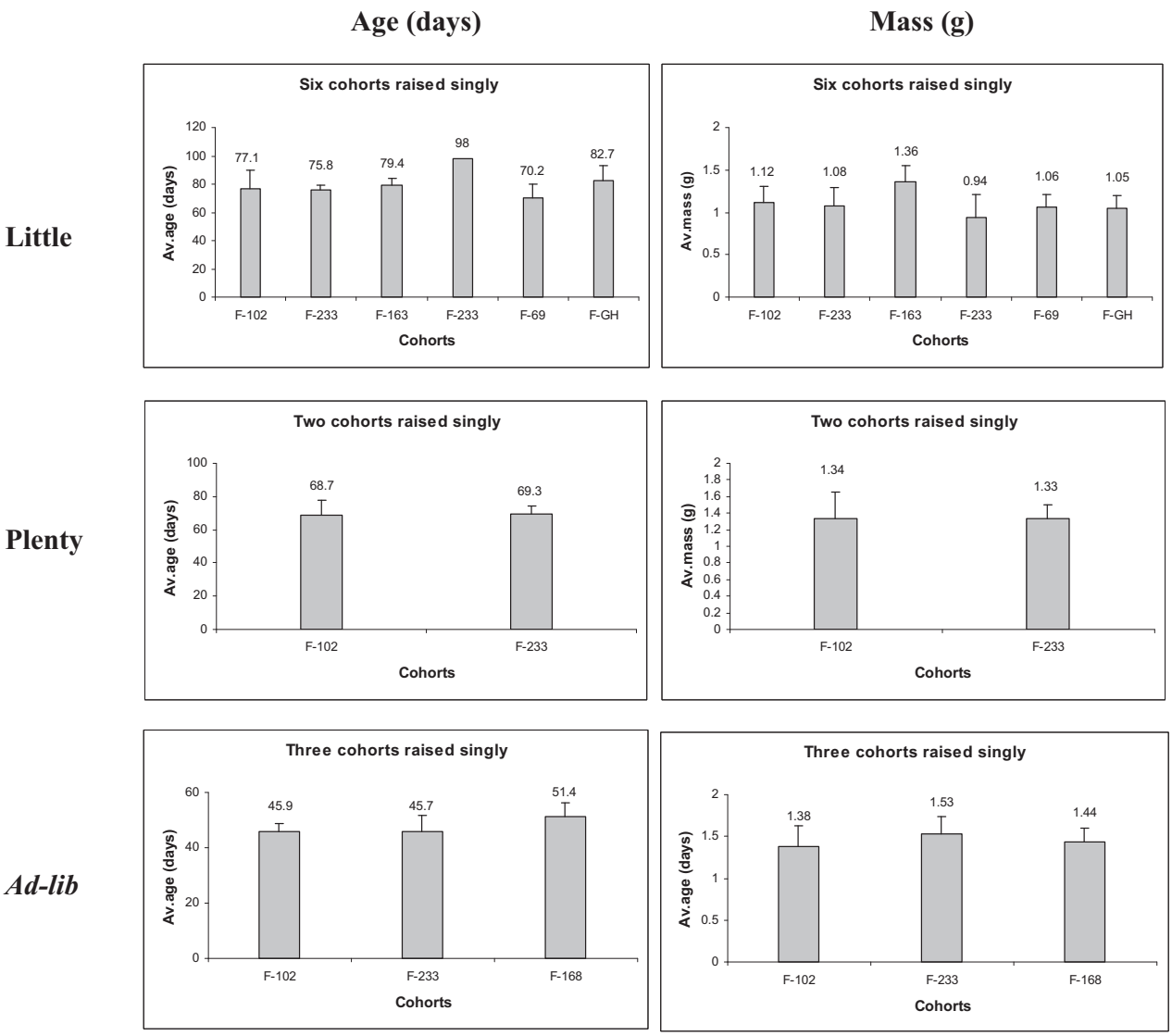


Fig. 1. – Average age and mass at metamorphosis of half-sib larval cohorts raised singly under different food regimes.

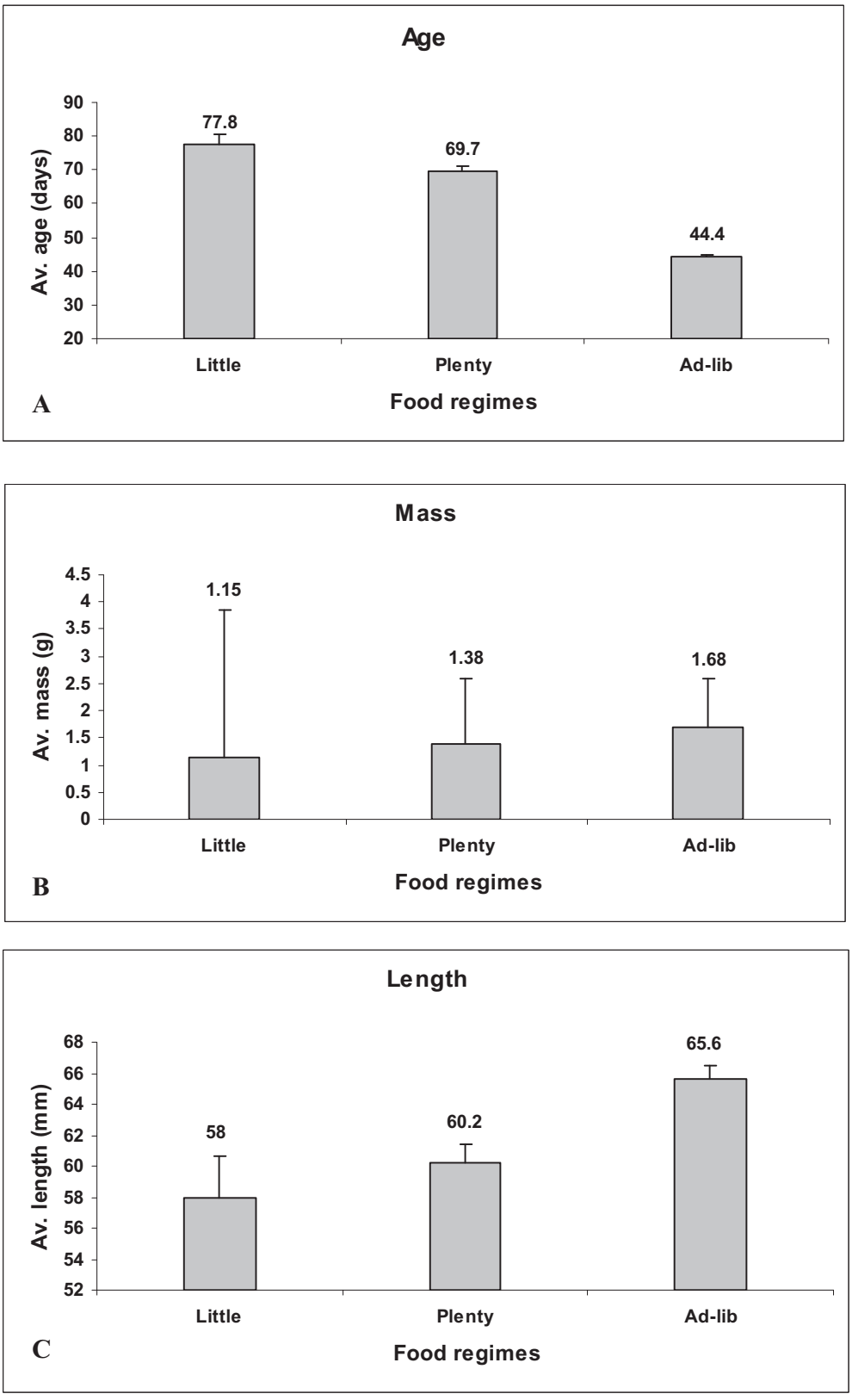


Fig. 2. – Average age, mass and length at metamorphosis when half-sib larvae belonging to a single cohort, were raised singly under three different food regimes.

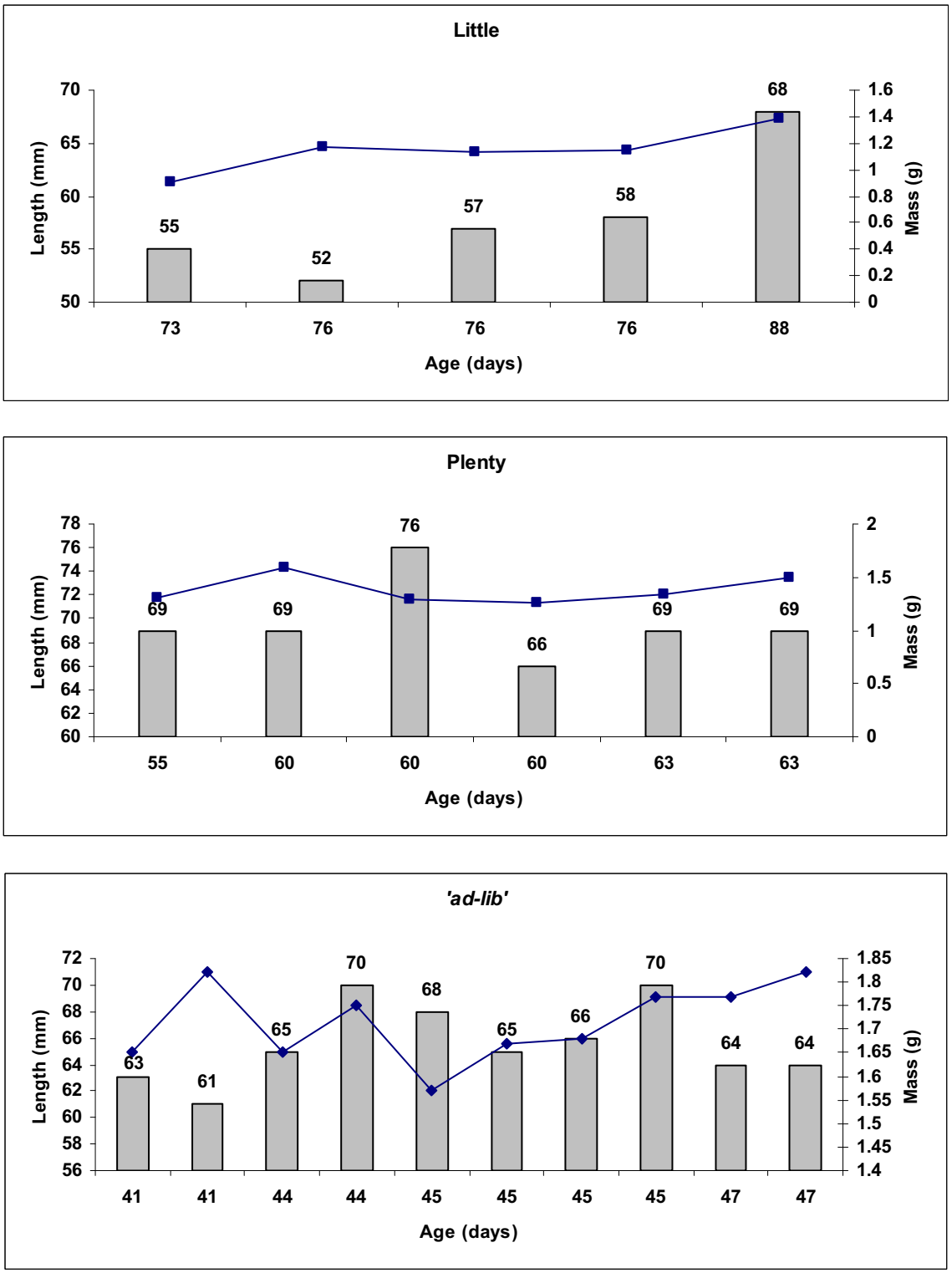


Fig. 3. – Relationship mass/age (line), and relationship length/age (column) at metamorphosis of half-sib larvae raised singly under different food regimes.

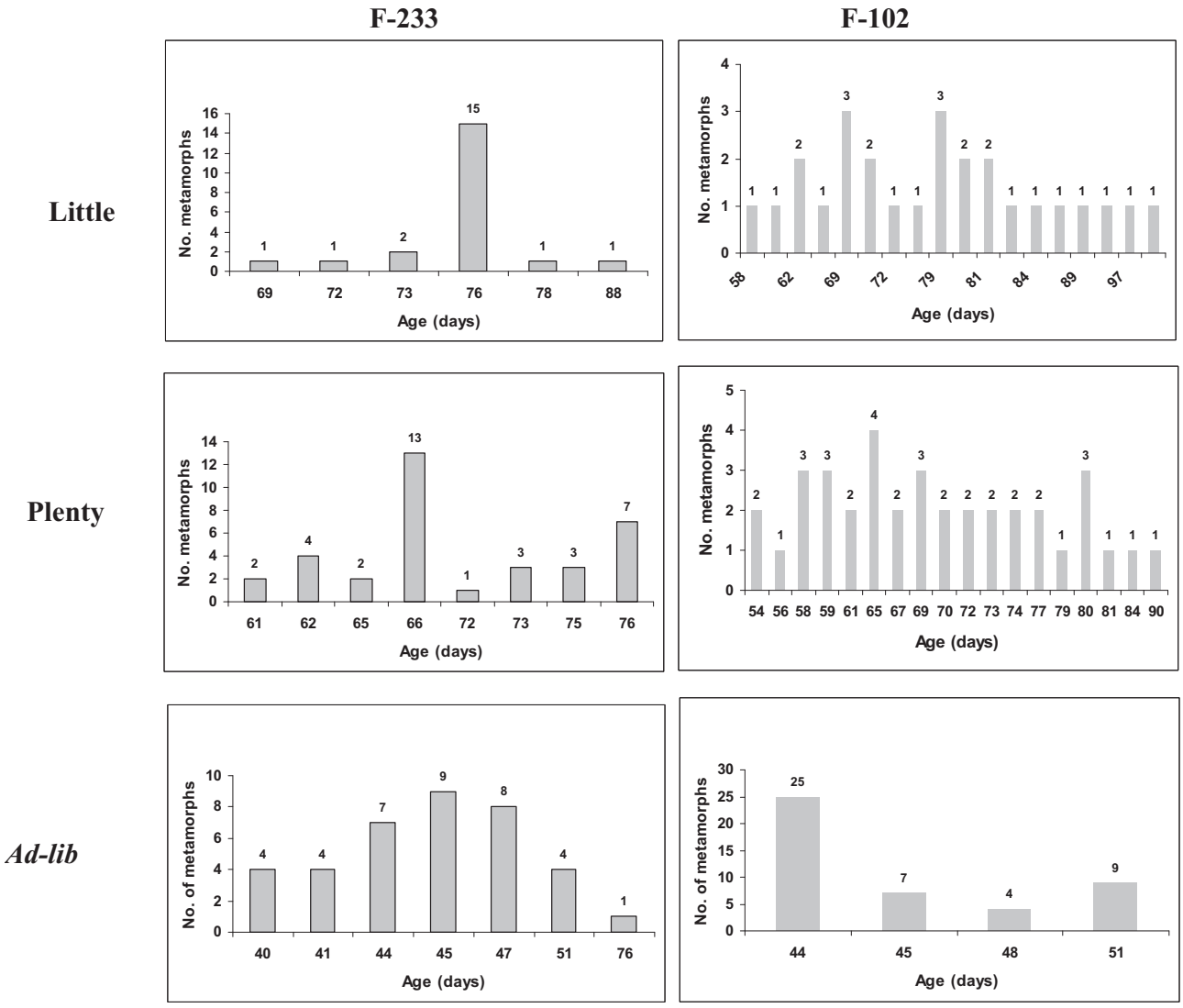
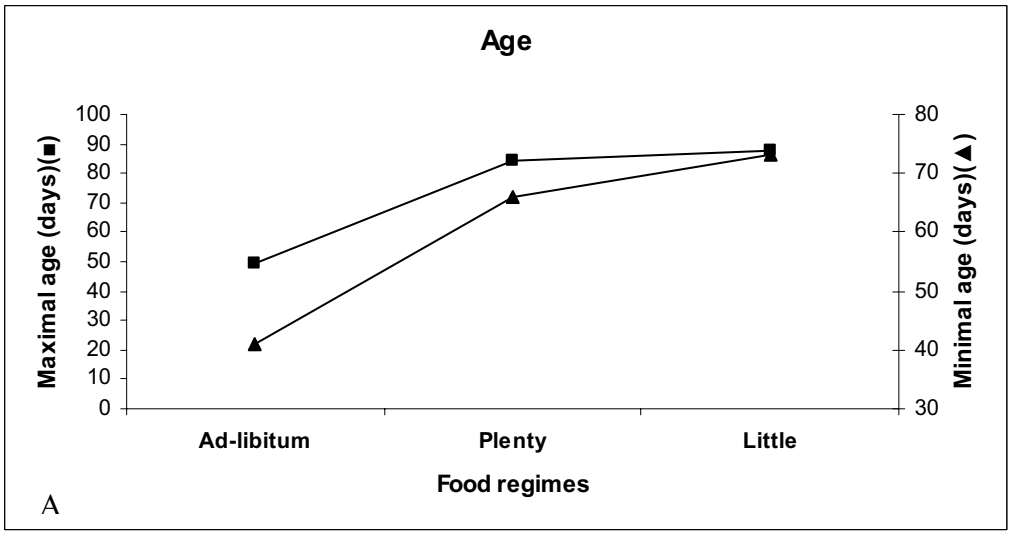
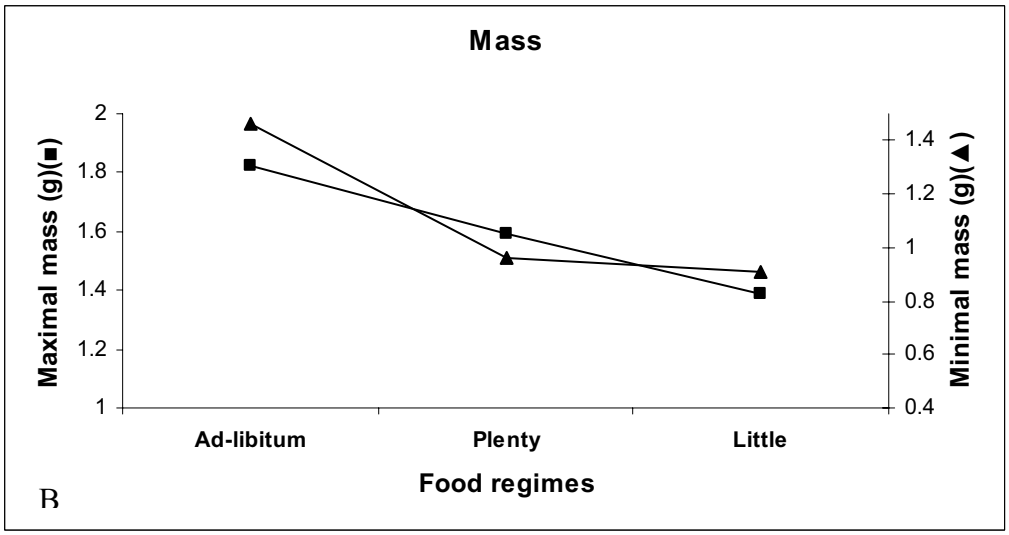


Fig. 4. – Number of metamorphs in two cohorts born on the same day and raised singly under different food regimes.





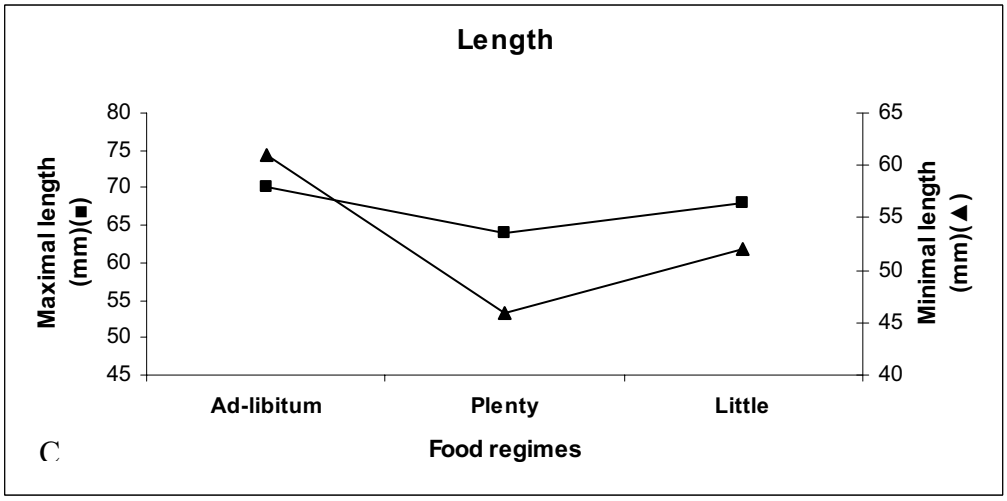


Fig. 5. – Maximal and minimal age, mass and length at metamorphosis in half-sib larvae raised singly under three different food regimes.

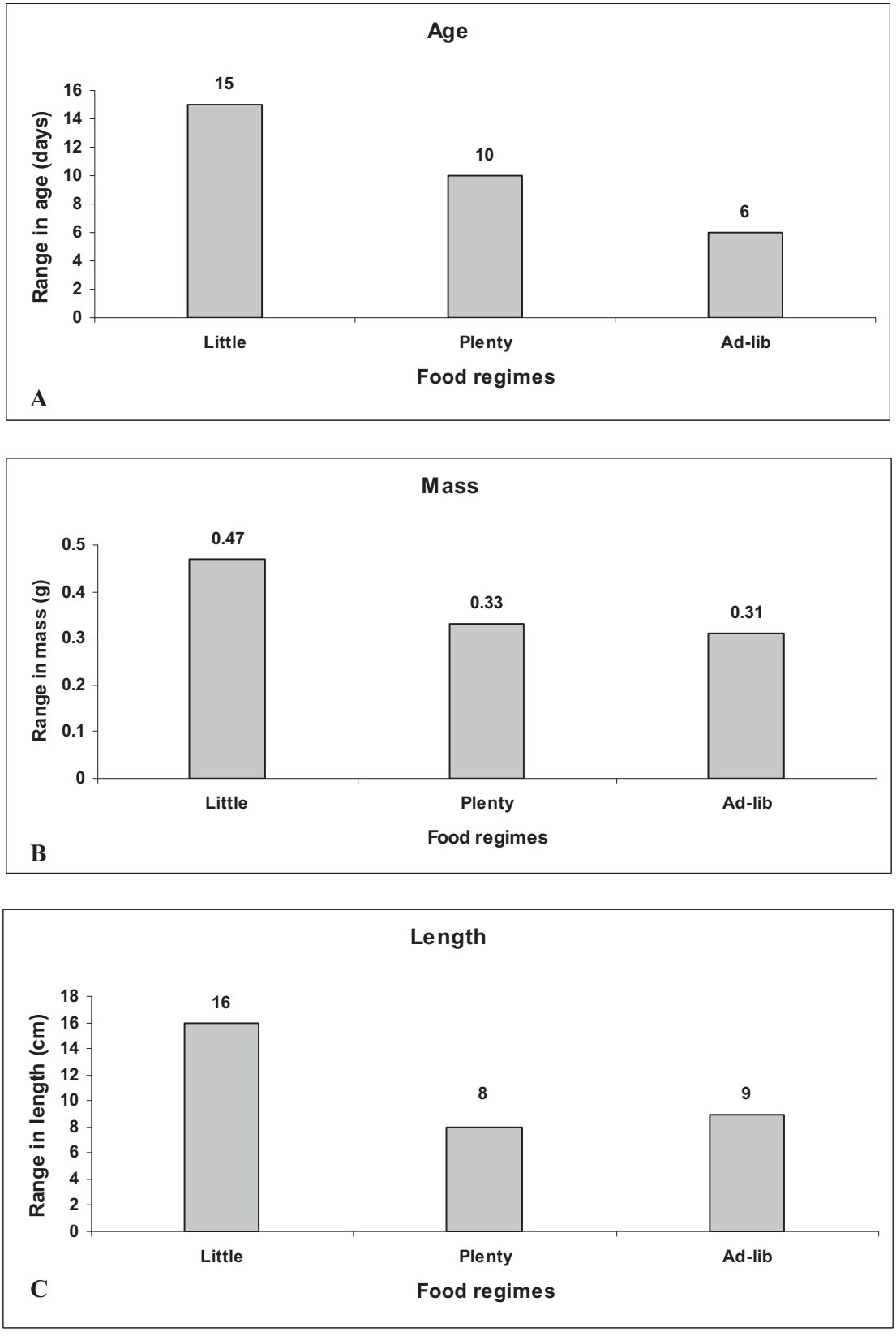


Fig. 6. – Range between maximal and minimal age, mass and length at metamorphosis.

DISCUSSION

Larval period and age of metamorphs

These are two different parameters: larval period is the time from when larvae are born or, alternatively, hatched from eggs laid. In the first case larval period will actually be their age, whereas in the second, larval period needs to be supplemented by the time it takes from the egg being laid to the time the larva hatches.

In some studies on this subject conducted on urodeles, age at metamorphosis was indeed known. Thus, *S. s. terrestris* females deposited their larvae in individual containers guaranteeing that larvae were half-siblings (KOPP & BAUR, 2000). In other studies, age can not be known with certainty since either larvae (LICHT, 1992; BRUNKOW & COLLINS, 1996; HICKERSON et al., 2005) or eggs (in *H. retardatus* OHDACHI, 1994, in *Desmognathus ochrophaeus* BEACHY, 1995, in *Ambystoma maculatum* WALLS, 1998, and in *H. retardatus* KOHMATSU et al., 2001) were collected in the field and hatched in the laboratory. Consequently, although the larval period is known, it is not identical with age, which remains unknown since the time eggs have been laid is not known. In both of these cases it can not be accurately known that the larvae are half-sibs since the egg batches may belong to more than a single female (communal egg-laying).

The effects of density and food resources on larval growth and thus on metamorphs

Most studies attempted to show effects of density (i.e. crowding) and resource (i.e. food) on time to metamorphosis and size of post-metamorphs.

HICKERSON et al., (2005) showed that metamorphic timing or the larval period in *Desmognathus quadramaculatus* was affected by food. In the same way, high food levels accelerated metamorphosis in *D. ochrophaeus* (BEACHY, 1995) and in *Hemidactylium scutatum* (O'LAUGHLIN & HARRIS, 2000).

Variations in *H. retardatus* larval growth affected both size and larval period (KOHMATSU et al., 2001). However, different food regimes under which larvae of *A. gracile* were raised did not differ in their effects on variation in mass at metamorphosis (LICHT, 1992). When larvae were raised on low and high food levels no significant differences could be detected in body mass at metamorphosiss between those raised with food continuously available (‘*ad-libitum’*) and those fed on low food levels (LICHT, 1992). Similarly, no effect on size at metamorphosis was observed in *Ambystoma tigrinum* (BIZER, 1978) nor did food regimes have any significant effect on days to metamorphosis in *D. quadramaculatus* (HICKERSON et al., 2005). In larvae of *Ambystoma opacum* raised on a high food level, a significant correlation was noted with size at metamorphosis (SCOTT & FORE, 1995).

The effect of food when larvae are raised singly with no density effect possible was studied in *A. tigrinum nebulosum* raised in individual containers on three food levels: high, medium and low (COLLIN & CHEEK, 1983). A similar study by KOHMATSU et al. (2001) on larvae of *H. retardatus* raised in solitude found no significant difference in size or days to metamorphosis.

Maximum and minimum, ranges and variations

Metamorphosis is not temporally fixed in time nor is it allometrically fixed in dimensions of metamorphs. There are larvae that take longer to metamorphose and attain smaller dimensions than others. WILBUR (1976) noticed very high variance in the same pond. Thus, variation in duration of larval period and body size at metamorphosis was attributed to density of conspecific larvae and time of oviposition. O'LAUGHLIN & HARRIS (2000) raised 120 larvae of *H. scutatum* under different feeding regimes studying mass at metamorphosis and larval period. Different food regimes caused some differences in larval periods. They noticed 4.6% difference in age and 26.7% differences in mass at metamorphosis. Such important individual variation can affect the adult population.

In the present study it was shown that both age and size of salamander larvae at metamorphosis is resource (food) dependant. In addition, marked temporal and allometric differences in metamorphs were noticed when larvae were raised under each food regime. Differences of over 30% were found in both age (32%) and mass (31.5%) at metamorphosis.

What can be the cause of such significant variations in age and size at metamorphosis? Since this variation is present in all experimental setups, the explanation may be that these half-sib larvae have a multi-paternal origin.

Multiple mating and sperm storage are both known in *Salamandra* and therefore sperm mixing may take place (discussed in WARBURG, 2009b). It was previously shown that there is variability in mass of new-born larvae within a half-sib cohort ranging between 5.1-10.1% (COHEN et al., 2005). This can be an outcome of multiple mating resulting in multi-parenthood. Since salamanders are known to be capable of storing sperm in the dorsal roof of the cloacal gland, the spermathecae, it is possible that certain sperm-mixing does take place in the uterus. As for sperm-storage, the fact that females arrive near the ponds in consecutive years (WARBURG, 2006) rules out the need to store sperm in their spermathecae since a salamander gets more than a single chance to mate and can mate every year.

This variability increased during larval growth because larvae used different growth modes (COHEN et al., 2006). Consequently, variation in dimensions more than tripled by the time these larvae metamorphosed.

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SHORT NOTES

The sponge-inhabiting barnacle *Acasta spongites* (Poli, 1795) (Crustacea, Cirripedia), a first record for the southern North Sea: how artificial habitats may increase the range of a species

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KEY WORDS : barnacle, North Sea, shipwreck, artificial habitat

Barnacle species of the genus *Acasta* Leach, 1818 are obligate symbionts mostly found on Demospongiae (Choristida, Axinellida, Poecilosclerida, Haplosclerida and Dictyoceratida) (1; 2) although some species are associated with Anthozoa (Alcyoniaria, Octocorallia and Antipatharia) (2; 3). Twenty seven species belonging to this genus have been described (2), most of them distributed in tropical, subtropical and warm temperate regions of the world. *Acasta spongites* (Poli, 1795) is the only known member of the genus occurring in northeast Atlantic waters.

The known geographic occurrence of *A. spongites* ranges from the British Isles (4-6) to the Mediterranean (4; 7; 8) through the Atlantic coast of France (9; 10). The species is apparently not reported from the North Sea (11). It is also mentioned in the Red Sea, Japan and South Africa (12) but these records may need confirmation. The occurrence around the British Islands is restricted to the south and southwest coasts only (13) and it is also noted on the south coast of Ireland (13). Overall, the number of recent records remains very limited. The earlier presence of the species in the English Channel was documented in The Plymouth Marine Fauna (14) and the species list of Glaçon (15). However, although recent records are rather scarce, the species is not uncommon in suitable habitats of the western Channel (own observations).

A. spongites is a sublittoral species living embedded in various species of Demospongiae (7; 16). In north-western European waters, *A. spongites* is invariably found associated with the sponge *Dysidea fragilis* (Montagu, 1818) (5; 6; 17; 18). The sponge and its host need hard substrates for their establishment, which is obviously a limiting factor for the spread of both species.

The Belgian Continental Shelf consists mostly of sandy sediments with some patches of pebbles in gullies between sandbanks (19). However, typical epifaunal species are present on the large number of shipwrecks that can be found in the Belgian marine waters (20-23). Until recently, these offshore hard substrates received little attention and their biota remained largely unknown. A recent investigation that studied the epifauna of four shipwrecks in Belgian marine waters was undertaken between 2003 and 2005 and revealed the presence of *A. spongites* on one of the sites. This shipwreck, the Kilmore, sank in 1906 and lies southwest of the Westhinder sandbank, 32km offshore (N051°23'.730 - E002°29'.790; WGS84) at a depth of 30m (LMWS). Sixty three quantitative samples (frames of 25x25cm) were scraped from the surface of the shipwreck. Two of these samples revealed the presence of a total of three specimens of *A. spongites* in samples taken at five meters above the bottom. The first specimen was found in March 2005. It measured 8.6mm (basal diameter), by 9.2mm (height from base to summit of the carina) (Fig. 1). The sizes of the two specimens found in August 2005 were 3.9x3.6mm and 3.0x3.2mm, respectively. Mature size given for this species in the literature is about 8mm in basal diameter (6; 7) indicating that at least the specimens from March can be considered as adults. They were all associated with the sponge *D. fragilis*. This sponge was identified on 13% of all the samples from the Kilmore and on 10% of all the samples from prospected Belgian shipwrecks (N=192). The fouling community of the Kilmore was dominated by the hydrozoan *Tubularia indivisa* Linnaeus, 1758, which covered most of the shipwreck surface. A more detailed description of the epifaunal communities on shipwrecks can be found elsewhere (20-23). Other barnacles found during the study of Belgian shipwrecks included *Verruca stroemia* O.F. Müller, 1776 and *Balanus crenatus* Bruguiére, 1789.

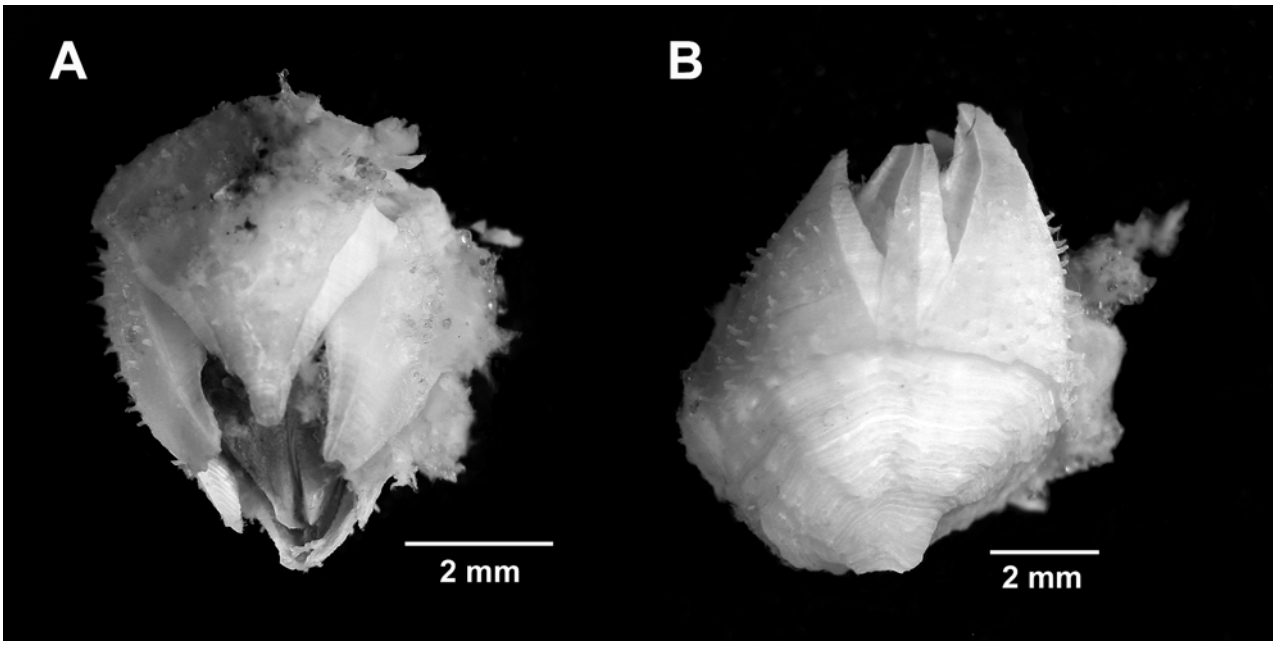

Fig. 1. – Photograph of *Acasta spongites* sampled on the Kilmore shipwreck. A: view of the operculum. B: lateral view.

This is the first record of *A. spongites* for Belgium and for the Southern Bight of the North Sea. It has not been found on other shipwrecks yet, but it is reasonable to think that a more intensive sampling effort would bring new records from other sites on natural and artificial hard substrates. Furthermore, *D. fragilis* was identified on other shipwrecks. Since *A. spongites* lives in a sponge that largely covers the barnacle, its presence may remain unnoticed to the unskilled observer.

Recently, other barnacle species with warm water affinities such as *Balanus perforatus* Bruguiére, 1789 and *Balanus amphitrite* Darwin, 1854 as well as more exotic species have been discovered in the waters off the southern North Sea (24, and Kerckhof unpublished records) and in the English Channel (25). This indicates that certain barnacle species are spreading to the north or establishing themselves as a response to recent climate changes, which are predicted to result in broad planktonic, pelagic and benthic community changes (26; 27). However, certain particular habitats were not thoroughly sampled in the past, so it is difficult to assess if the presence of a certain species with southern affinities might be the result of a range extension, due to the recent warming up, or if it was present over a longer period of time and overlooked/neglected in the past.

The fauna of the eastern part of the English Channel can be considered as an impoverished version of the western part. Many species present in the western English Channel are apparently absent in the eastern part while all species present in the east are generally present in the west (28). This distribution pattern of species is thought to be mostly a result of a temperature gradient with warmer and less fluctuating temperatures in the western English Channel than in the eastern part. However, the absence of suitable habitat may also prevent the larvae of some species that need hard substrates from surviving long enough to find an appropriate settling place. Hard substrates are indeed available for the settlement of epifaunal species around the Dover Strait because of the strong currents, which prevent sedimentation in this particular area (29; 30). However in the areas immediately adjacent to the Dover Strait, only isolated patches of natural hard substrates remain. This strongly limits the probability for larvae of epifaunal species to find a suitable settling place. Our finding is an illustration of the fact that so-called southern epifaunal species can penetrate into the southern North Sea and survive, provided that suitable habitat, such as shipwrecks, is present.

Until recently, hard substrates in Belgian marine waters received little attention due to their limited presence and the practical problems of sampling them. Nonetheless, our findings support the view that certain particular and rare/uncommon habitats such as hard substrates, whether artificial or not, might act as stepping stones and thus enhance the further spread of certain warm water species limited to hard substrates further into the North Sea. It should be noted that the sponge itself, *D. fragilis*, has only been recently recognized as occurring in the southern North Sea (20).

In Belgian marine waters, *A. spongites* lives under the influence of Atlantic water masses that pass through the English Channel. Generally, the residual current runs from southwest to northeast (31). This ensures a high salinity and a steady supply of larvae. In the region of the Hinderbanks, other species with southern affinities (molluscs, bryozoans) have recently been found and were previously unknown to the southern North Sea (23; Houziaux; Kerckhof unpublished data). Further to the north, in the Dutch and German part of the North Sea, similar regions occur with patches of natural hard substrate and shipwrecks that might be suitable for the establishment of a whole suite of epifouling species with southern affinities. However, all these particular habitats have been and still are incompletely known, which ham-

pers the ongoing discussion on the possible effects of climate change.

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Low genetic differentiation between populations of *Podarcis sicula* (Reptilia, Lacertidae) from the Italian islands off the coast of Campania and the mainland

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The Italian wall lizard, *Podarcis sicula* (Rafinesque-Schmaltz, 1810), is a morphologically variable species, widespread in Italy (Sardinia, Sicily and most of the Tyrrhenian and Italian Ionian and Adriatic islands included), Corsica and the northern part of the east Adriatic coast (Slovenia, Croatia and many Dalmatian islands and part of Montenegro). Naturalized populations are found in Spain, Portugal, France, Turkey and USA (1). It is found on multiple small islands throughout its range, most of which contain morphologically distinctive populations. This has led to many subspecies being described. HENLE & KLAVER (2) reviewed 91 described subspecies and accepted 52, of which 47 were single island endemics. Assessment of the genetic distinctiveness of these subspecies is essential, since although the peninsular populations are generally not threatened, island populations may be vulnerable (1). Some microinsular populations may have gone extinct through environmental degradation, while a distinct subspecies, *P. s. sanctistephani* of Santo Stefano Island (Arcipelago delle Pontine), appears to have been replaced after the nominal subspecies was introduced to the island (3; 4). PODNAR et al. (5) identified six main haploclades within *P. sicula*. One of these, the "Monasteraace group" is known from only one locality on the Ionian coast, indicating that extensive sampling is needed to fully determine genetic diversity. However, little diversity was found between some Adriatic island subspecies and mainland forms, corroborating doubts of the validity of several subspecies.

Our aim was to examine genetic diversity of *P. sicula* from various Campanian islands and the South-eastern Pontian Archipelago islands, some of which have been described as island endemic subspecies. This area lies on the border of the area where the "campestris-sicula" haplogroup was found (5), but no previous information was available regarding the majority of these island populations, only one individual from one island, Ischia, had previously been determined. We examined diversity by sequencing part of the mitochondrial DNA cytochrome b, so that our results could be compared to previously published data on *P. sicula* (5), but also so that levels of diversity could be compared to other insular *Podarcis* subspecies, such as those from the Balearics (6).

TABLE 1

Localities of samples used, their position on Fig. 1, and their respective haplotypes in Fig. 2

Code	Locality	Map Code	Haplotype
24	Ventotene	1	B
20	Ventotene	1	I
31	Santo Stefano	2	G
25	Santo Stefano	2	J
44	Ischia	3	A
45	Ischia	3	E
CA 4	Ischia	4	A
Pr 14	Procida	5	D
51	Procida	5	A
PP 3	Punta Pennata	6	A
Ni 3	Nisida	7	A
Ni 4	Nisida	7	H
Na 4	Napoli	8	B
Rv 4	Rovigliano	9	B
Vt 1	Vetara	10	A
Vt 2	Vetara	10	A
62	Castelluccia	11	F
71	Gallo Lungo	12	A
32	Capri	13	A
43	Capri	13	C
Li 9	Licosa	14	K
Li 10	Licosa	14	K
74	Camerota	15	B

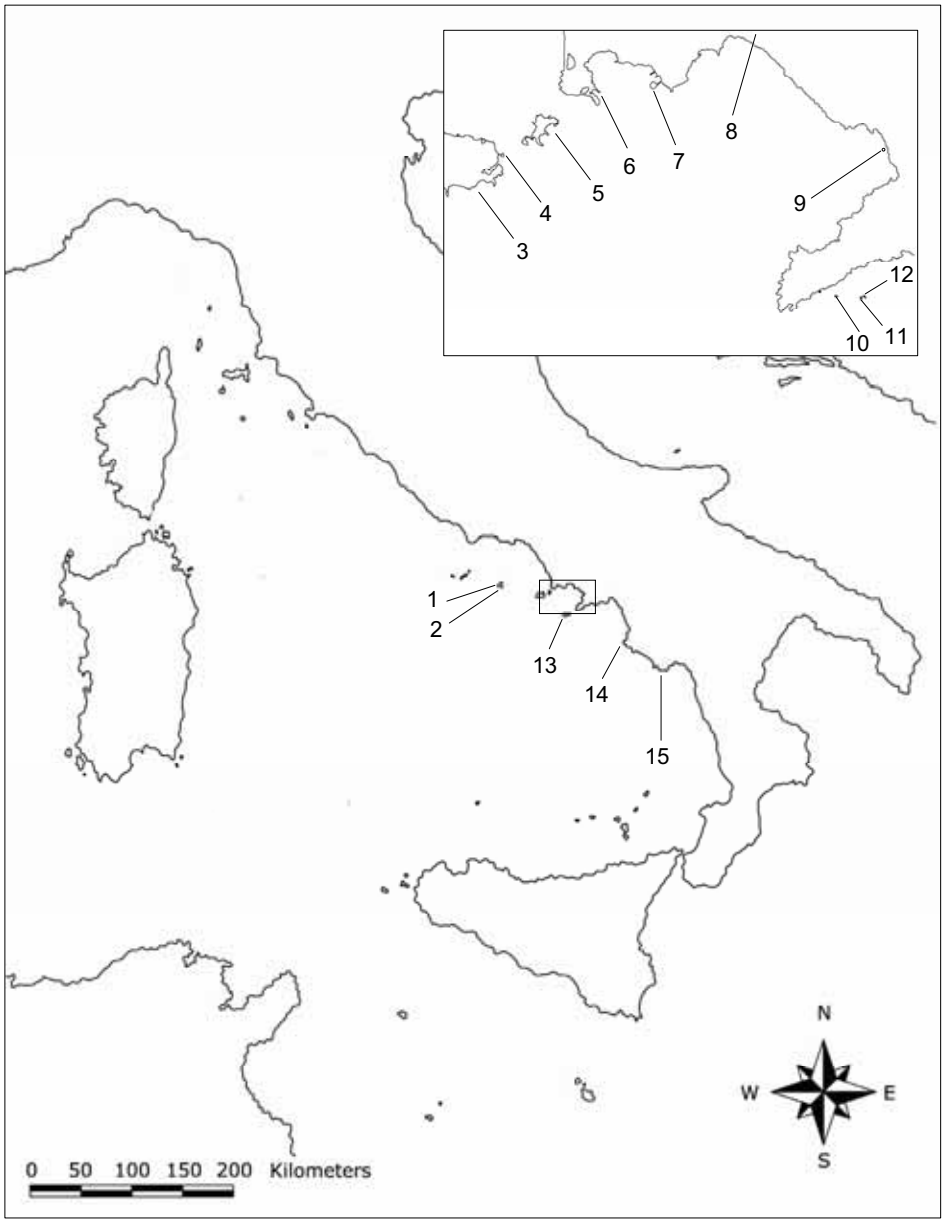

Fig. 1. – Map showing the sampling locations of *P. sicula* sequenced for this study. Codes are given in Table 1.

The number and geographic locations of the specimens used in this study are given in Table 1 and Fig. 1. Individuals were released after tail tips were collected. The tissue samples have been collected under the permits of the Ministero dell'Ambiente e della Tutela del Territorio e del Mare, DPN-1701/2006 and the Riserva Naturale Marina di Ventotene e Santo Stefano, Ente Parco Regionale dei Campi Flegrei, Ente Parco Nazionale del Cilento e Vallo di Diano.

Tissue samples were stored in 100% ethanol. Total genomic DNA was extracted from tissue samples following the SAMBROOK et al. (7) protocol. Polymerase Chain Reaction primers used in both amplification and sequencing were GluDG and Cytb2 from PALUMBI et al. (8) and KOCHER et al. (9) respectively. Amplification conditions were the same as described by HARRIS et al. (10). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Mitochondrial DNA sequences were aligned by eye. Within species, when variation is low, networks are a more appropriate way of assessing genetic variation than using the more common phylogenetic tree building algorithms (11). Since initial inspection of the sequences indicated that variation was low, all the haplotypes were joined in a most parsimonious network (Fig. 2).

In total, 23 new specimens were included for a total of 390 base pairs. Five closely related individuals from GenBank, with three distinct haplotypes were also included, and assigned the same codes as in the original publications (12; 5). Alignment was facile as this is a protein coding gene, and no insertions or deletions were needed. New haplotypes have been submitted to GenBank with accession numbers EU916814 to EU916824. In total 11 new haplotypes were recovered that differed by at most four nucleotide substitutions across this region.

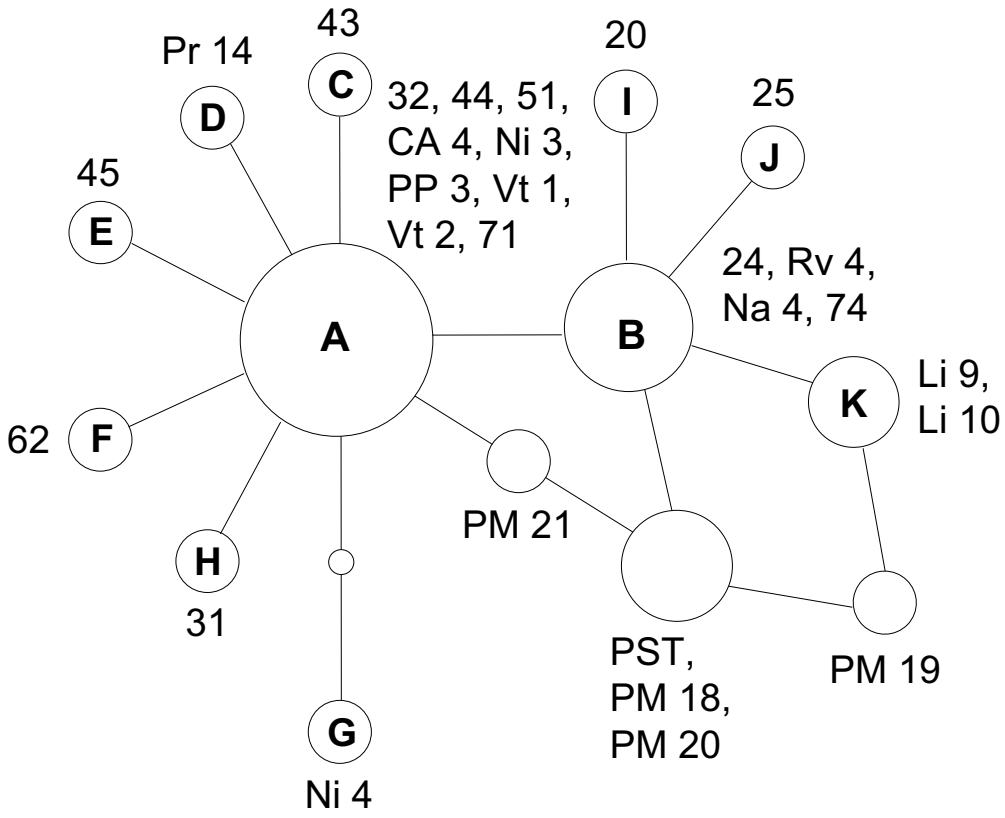

Fig. 2. – Most parsimonious network of variation of the 11 new haplotypes (A-K) plus three closely related haplotypes from GenBank (PM21, PM19 and individuals PST, PM18 and PM20). Size of circles indicates relative number of individuals with that haplotype. The small circle between haplotypes A and G indicates the sole presumed missing haplotype. Individual codes are given in Table 1.

Our results show that the island populations are genetically very similar to each other and to the mainland populations, representing the "*campestris-sicula*" haplogroup of PODNAR et al. (5). The two commonest haplotypes, A and B are found on several islands and on the continent, while the remaining rare haplotypes are generally island specific. This is despite morphological differences, such that some island forms have been assigned subspecific status, such as *P. s. ventonensis* from Ventotene, *P. s. klemmeri* from Licosa and *P. s. gallensis* from Gallo Lungo and Castelluccia. Our results suggest that these differences are more likely due to founder effects, drift phenomena and rapid adaptations to local island condition rather than longer term evolutionary isolation. All of these islands, except for Ventotene and Santo Stefano, lie in relatively shallow water (less than 100m), and so would have been connected to the continent during sea level fluctuations associated with the last glacial cycles.

Despite the low levels of differentiation, overall diversity was not particularly low, with 11 haplotypes determined from 23 individuals. This implies that during the last glacial period populations of *P. sicula* were large enough in this area to maintain considerable diversity, something that is not surprising given that populations of *Podarcis* in the southern Iberian Peninsula and North Africa were also relatively large (13). Moreover, since the Olocene the Campanian islands vertebrate fauna seem to be related mainly to human intervention rather than to natural colonization (14). During the XX Century, for example, on Gallo Lungo Island, some continental lizard individuals have been introduced to control insect population (G. Casa, pers. comm., 16-05-2007). The "star-like" network configuration is also consistent with a population expansion, presumably following the last glacial maxima. At this time the islands were colonized, and drift effects led to the current morphological differences. CAPULA & CECCARELLI (15) also reported low diversity between Pontine Archipelago populations of *P. sicula* and mainland populations based on allozyme electrophoretic data. A similar situation occurred in the colonization of the Berlengas islands off the West coast of Portugal by individuals of *Podarcis carbonelli* (16) that are morphologically distinct from their coastal relatives (17). Similar low-differentiation of microinsular populations is also seen in *P. tiliguerta* on islands off Sardinia (18) and in *P. lilfordi* in the Balearics (6). Our results thus further support the view of PODNAR et al. (5) that most of the island endemic subspecies of *P. sicula*, and perhaps of many other species of *Podarcis*, are not genetically distinct entities. However, this is not always the case, the endemic *Podarcis* from the Aeolian Islands once thought to be a subspecies of *Podarcis wagleriana* is now considered a distinct and critically endangered species, *Podarcis raffonei*, after genetic and ecological investigations (19). Considerable cryptic variation within *Podarcis hispanica* has also been reported (20, 21). Thus other island forms of *Podarcis* still need to be evaluated in case other unidentified genetically-distinct lineages exist.

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Occurrence of the invasive crayfish *Procambarus clarkii* (Girard, 1852) in Belgium (Crustacea: Cambaridae)

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The red swamp crayfish or Louisiana crayfish *Procambarus clarkii* (Girard, 1852) originates from north-eastern Mexico and the southern part of the USA, from Texas to Alabama and northwards to Illinois (1). Mostly due to its commercial value, this species has been introduced into other parts of the USA. Its range now includes the east and west coasts and northward up to the states of Idaho and Ohio (2). Nowadays, *P. clarkii* has been introduced almost worldwide. In Europe, it was introduced for the first time into southern Spain in 1973 for aquaculture, as bait used by anglers and for aquarium purposes (3). It was subsequently introduced into Portugal, Cyprus, the United Kingdom, France, Germany, the Netherlands (3) and recently Switzerland (4). The species is now present in at least 13 European countries and also occurs on islands like the Azores and the Canaries (5). Since its introduction, this species has given rise to naturally-breeding populations and soon became established in the wild. Recently, *P. clarkii* has been reported in a number of ponds and streams in the Netherlands, especially in the west of the country, where it rapidly expanded towards the Belgian border, but with only a few records in the east (6).

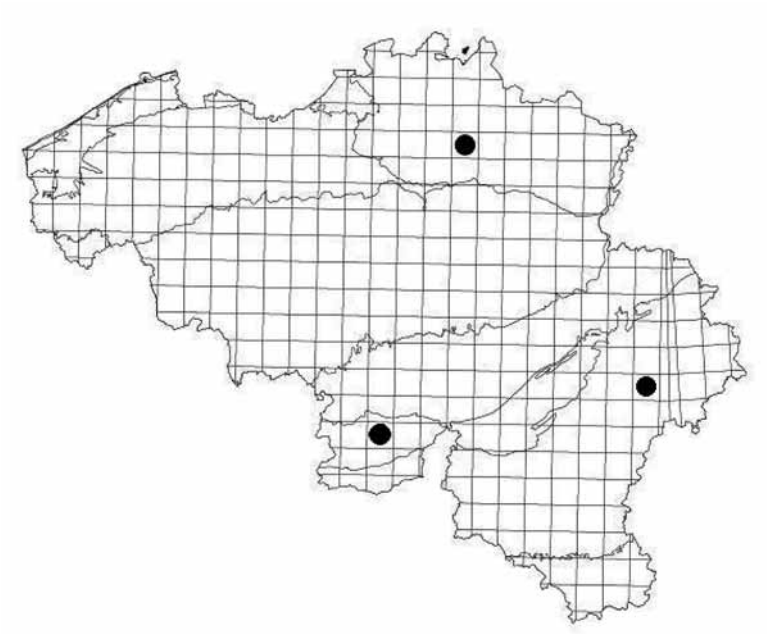

Fig. 1. – Distribution of *Procambarus clarkii* in Belgium, on a 10*10km UTM grid.

In Belgium, the first specimen of *P. clarkii* was found dead in the reservoir of Vielsalm (Ardennes, alt. 375m; Fig. 1) during the first Belgian crayfish distribution survey, made by the Station de Recherches Forestières et Hydrobiologiques of Groenendaal during the years 1983-1985. This specimen might have originated from a nearby restaurant. More interesting was the discovery of a living individual in a pond nearby Cerfontaine (Fagne-Famenne, alt. 245m; Fig. 1) on 3 September 1996 during a large scale distribution survey of crayfish in Wallonia, funded by a grant from the Ministry of the Walloon Region during the years 1994-1996. More recently, on 2 July 2008,

P. clarkii was found in the nature reserve Zammelsbroek in Zammel (Kempen, alt. 15m; Fig. 1). On 28 January 2009, populations of *P. clarkii* were found there in three ponds situated northeast of the nearby River Grote Nete although the ponds were not directly connected to this river. The ponds were sampled using a handnet with a mesh size of 0.5mm. Without much effort, several individuals including juveniles were caught, indicating that the species is well established and that it reproduces. The nature reserve Zammelsbroek has a varied landscape consisting of landdunes, meadows, shrubbery, ponds and brooks and is classified as biologically valuable (7). The presence of sensitive taxa such as caddisflies and mayflies indicates that the ponds at Zammelsbroek have a good biological water quality.

It has to be mentioned that the presence of *P. clarkii* has been suspected in the Belgian upper courses of the Rivers Leie, Scheldt and Sambre. These canalized rivers are connected by canals to the French River Somme. On 11 July 1997, many individuals of *P. clarkii*, including juveniles, were found together with *Orconectes limosus*, in the locality of Corbie-sur-Somme (France), in a pond closely connected to this river. Since then, some investigations have been done in order to check the presence of *P. clarkii* in the Rivers Leie, Scheldt and Sambre nearby the French border in the Province of Hainaut, but without positive results.

The scattered distribution of *P. clarkii* in Belgium (Fig. 1) suggests that the species probably escaped from nearby private ponds or was deliberately released by amateurs keeping crayfish as a hobby. It is also possible that the species has actually a much larger distribution but remained unnoticed, however, this seems unlikely as it is a large, conspicuous species. Regardless of its way of introduction, the expansion of the species in the neighbouring countries indicates that the species will become more common in Belgium as well and therefore, that more records are to be expected in the future.

Identification of *P. clarkii* is easy. Like other members of the family Cambaridae, it possesses a strong spur at the inner side of the carpopodite (Fig. 2). Moreover, the propodite is armed with strong spines on its inner side as well as conspicuous knots on its dorsal face (Fig. 2). The branchiocardiac grooves of the carapace converge dorsally. Lateral spines or tubercles in front of and behind the cervical groove are absent or reduced. The rostrum is devoid of a median keel and has an obvious triangular shape, the sides tapering anteriorly. The head itself is elongated and narrowing towards the front. Special attention is needed to identify juvenile crayfish, which are not coloured red and look very similar to other *Procambarus*-species (i.e. marbled crayfish) (6).

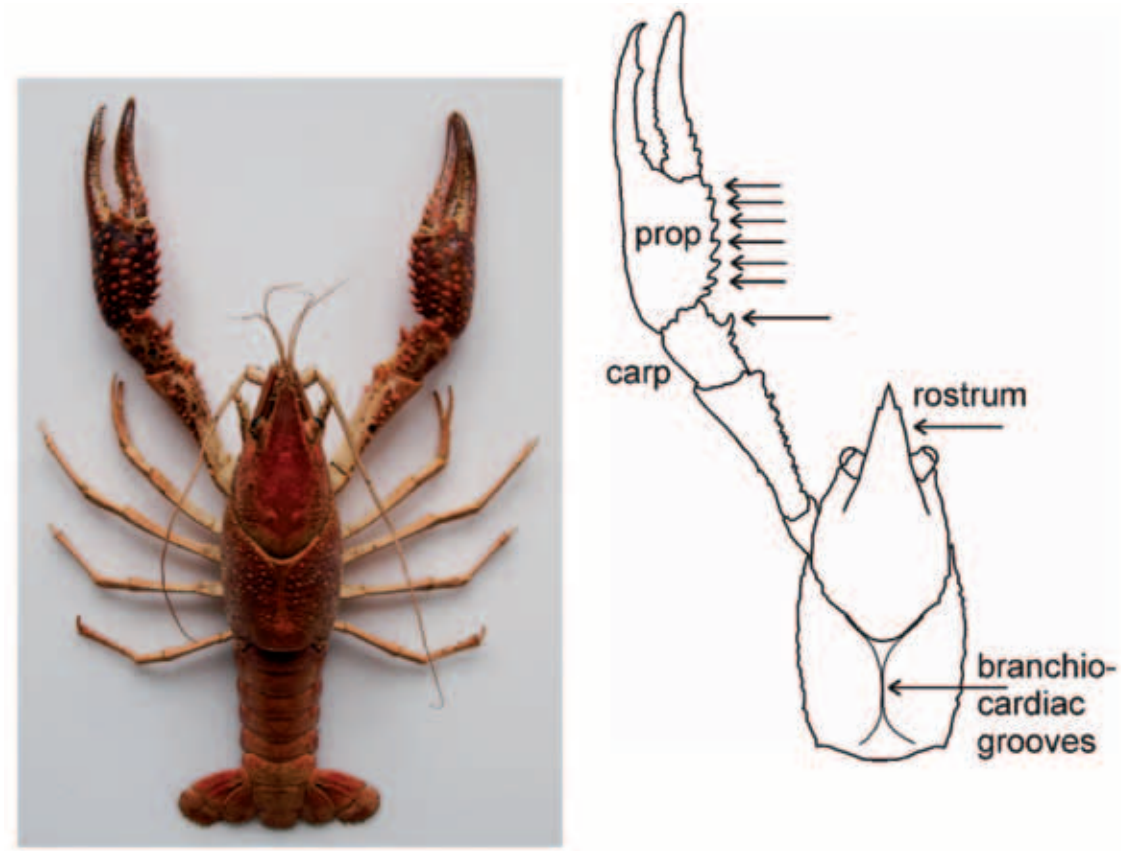

Fig. 2. – Photograph of a naturalized *Procambarus clarkii* specimen (left) and drawing of typical (arrowed) morphological features (right). (prop: propodite; carp: carpopodite).

Besides the red swamp crayfish, the family Cambaridae is also represented in Belgium by the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), an invasive species that was reported for the first time in 1962 from the River Meuse (8). Five other Cambarid species that have been introduced in Europe, have not yet been reported from Belgium: *O. immunis* (Hagen, 1870), *O. juvenilis* (Hagen, 1870), *O. virilis* (Hagen, 1870), *P. acutus* (Girard, 1852) / *zonangulus* (Hobbs & Hobbs, 1990) and an unidentified species of *Procambarus*, which is known as the marbled crayfish (9; 10). All these Cambaridae were imported from North America and are liable to appear one day in our country. Crayfish belonging to the family Astacidae are also represented by three species in Belgium: *Astacus astacus*, *A. leptodactylus* and *Pacifastacus leniusculus*. *A. astacus* is the only indigenous species in Belgium, however, its distribution area has dramatically collapsed since the introduction of exotic crayfish carrying the crayfish plague (8). *A. astacus* is now restricted to a few places in the south and the east of Belgium (11). *A. leptodactylus* was probably introduced in the 1950's and *P. leniusculus* in the 1970's, both for alimentary purposes (8).

P. clarkii is considered as an important polytrophic consumer that may act as a keystone species (12). The species is omnivorous and consumes mainly microbial-enriched detritus, benthic and planktonic invertebrates and plant material (13). Research about the feeding habits indicated that there was a seasonal difference in food choice: plants were mostly consumed during summer, while prey were especially consumed during winter (12). Besides this seasonal pattern, the species also showed a difference in food selection depending on age and maturity. *P. clarkii* is a large, aggressive species that is well adapted to areas with drastic seasonal fluctuations in water level, where it survives by digging deep burrows (14). Individuals are able to spread over land and are in this way not restricted to the aquatic environment to colonize new areas. The species tolerates low oxygen concentrations and strong fluctuations in salinity and acidity (13). In addition, it is highly resistant to the crayfish plague, has an early maturity, a rapid growth rate and a high fecundity. The red swamp crayfish is known to contribute to biodiversity losses and habitat degradation recorded in several freshwater systems of south-central Europe (15). The burrowing activity of this species may result in the destabilization of banks of rivers and lakes. An indirect effect of the crayfish activity may be the increase in turbidity, leading to a decrease in light penetration and thus a decrease in plant growth. As *P. clarkii* feeds especially on molluscs, fish, amphibians, macroinvertebrates and macrophytes, it may cause changes in food webs and even disappearance of some species (14). Consequently, it can be expected that this opportunistic species will have a strong impact on the local community.

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