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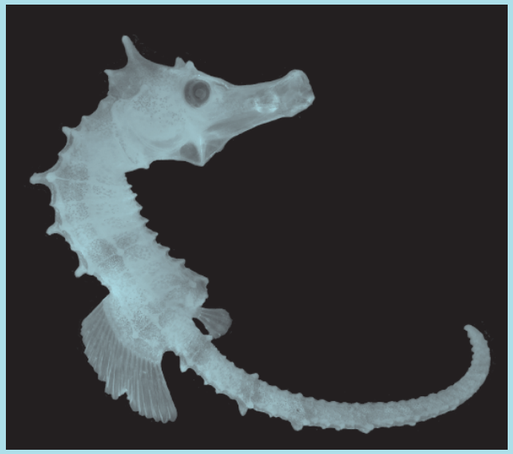

BELGIAN JOURNAL OF ZOOLOGY

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Mechanisms regulating functional monogyny in a Japanese population of *Leptothorax acervorum* (Hymenoptera, Formicidae) : dominance hierarchy and preferential egg cannibalism

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ABSTRACT. Queen behaviour of *Leptothorax acervorum* collected in northern Japan was observed in four functionally monogynous colonies (one functional mated egg-laying queen with some supernumerary mated but sterile queens) and two monogynous colonies with some virgin queens. In three functionally monogynous colonies, dominance behaviour including antennation, biting, pulling, and remarkable avoidance where queens fled approaching the functional queens, were frequently observed among queens. In two of the three colonies, an almost linear dominance hierarchy was established among queens and only the top ranked queen laid eggs. However, the hierarchy was not stable : in one colony the queens in second and third ranks and the second ranked queen in the other colony were expelled from colonies. Such queen antagonism was very rare in the other functionally monogynous colony, where workers expelled the fertile queen, and the other queen replaced the egg-layer. In monogynous colonies with virgin queens, virgin queens showed worker-like behaviour, and dominance interactions completely lacked among queens. Oophagy was often observed among nestmates : this always occurred just after oviposition. Eggs of functional queens were not eaten while most eggs laid by supernumerary queens were eaten by functional queens and workers. Supernumerary queens never showed oophagy. Workers laid trophic eggs and reproductive eggs : the former ones were always eaten while one third of the latter survived.

KEY WORDS : dominance hierarchy, ants, functional monogyny, oophagy.

INTRODUCTION

In ants, many species show polygyny in which there are several functional queens per colony (HÖLLDOBLER & WILSON, 1990). Polygyny in mature colonies is usually a secondary polygyny where new queens are added by adoption into an already existing monogynous colony (HÖLLDOBLER & WILSON, 1990). In general, secondary polygyny maintains the reproductive function of most queens without aggressive display among them (BOURKE & FRANKS, 1995). However, some ant species show a remarkable reproductive skew among coexisting queens. The most remarkable case is "functional monogyny", in which only one mated individual lays eggs while the other inseminated individuals are sterile (e.g. BUSCHINGER, 1968). This social structure is known from seven Formicoxenini, and the queenless ponerine *Pachycondyla* sp. (BUSCHINGER, 1968, 1990; ITO, 1990, 1993). Functional monogyny is proximately regulated by antagonistic behaviour leading to dominance hierarchy among nestmates in *Leptothorax* sp. A., *L. gredleri* Mayr, 1855 and *Pachycondyla* sp. (HEINZE & SMITH, 1990; HEINZE et al., 1992; ITO, 1993).

To know how and why such high reproductive skew exists is very important for understanding the evolution of social life in animals (reviewed in REEVE & KELLER, 2001). *Leptothorax acervorum* Fabricius, 1793 is an interesting subject for understanding this problem, since its social structure varies geographically : in central, western and northern Europe, many colonies show functional polygyny, i.e. several egg-laying queens occur per colony (BUSCHINGER, 1968; BOURKE, 1991; STILLE et al., 1991; HEINZE et al., 1995) while in northern Japan and central Spain, colonies having multiple mated queens always show functional monogyny (ITO, 1990; FELKE & BUSCHINGER, 1999). In Alaska, both functional monogyny and functional polygyny were found in the same population (HEINZE & ORTIUS, 1991). In this paper, I demonstrate the mechanisms regulating functional monogyny in a Japanese population of *Leptothorax acervorum*, and compare the results with studies in other populations (BOURKE, 1991; HEINZE & ORTIUS, 1991).

METHODS

The ants

Leptothorax acervorum is a common holarctic ant, distributed through Europe, Asia and northernmost North America. In Japan, the ants are found in Hokkaido and mountain areas of Honshu and Shikoku (TERAYAMA et al., 1992). In Furano, northern Hokkaido, 50% of the colonies have multiple dealate queens per colony, however, there is only one principal egg layer having well developed ovaries even if there are multiple mated queens (ITO, 1990). A few colonies have additional egg-laying queens;

however, the ovaries of these queens are distinctively less-developed than those of the principal egg layer. In this paper, I use the term “functional queens” for principal egg layers and "supernumerary queens" for the non-reproductive mated queens and mated queens who lay eggs at a very low rate, and “virgin queens” for unmated queens.

The colonies for the present paper were collected from dead twigs fallen on sunny rocky outcrops in Shikaoi, eastern Hokkaido, northern Japan, in mid May (colony code A, B, C, D) of 1992 and early July (E, F) of 1990. This collection site is located ca. 50 km east of Furano. In the colonies collected in early July (colonies E and F), one queen per colony already had a swollen abdomen and there were several eggs in the nests. The colonies collected in mid May had neither such queens nor eggs. The numbers of dealate queens and workers in these colonies are shown in Table 1. Colonies C and D had only one functional queen and some virgin queens. The remaining four colonies had one functional queen with a few virgin queens and/or multiple supernumerary queens (functional monogyny).

TABLE 1

Composition of *Leptothorax acervorum* colonies observed in laboratory. Numbers in parentheses show duration of observation only for egg laying activity. All colonies had only one principal egg layer each.

Colony code	No. individuals				Dates observed	Hours observed
	Queens			Worker		
	Total	Mated	Virgin			
A	6	5	1	48	June 15 – July 15	44 + (24)
B	5	5	0	23	May 28 – July 12	72 + (24)
C	10	1	9	45	June 10 – 29	20 + (24)
D	5	1	4	30	June 17 – July 12	13 + (24)
E	7	6	1	36	July 2 – 10	18
F	7	7	0	29	July 8 – 22	16

Laboratory observation

The colonies were kept in an artificial nest measuring 14.5 x 8.0 x 3.0 cm at room temperature condition (ca. 20 ~ 25 °C). The bottom of each nest box was covered with plaster and brood chambers (2 x 6.5 x 0.5 cm) were excavated in the plaster floor. The chambers were covered with glass plates. Small mealworms and diluted honey were given as prey every day. All dealate queens were individually marked by enamel paint. In four colonies (A-D), observation started from just after the winter season (late May to mid June). These colonies were kept for one to two months and observation ended in the egg-laying season. For the other two colonies (E and F), behavioural observation started during the egg-laying season in early July.

Behaviour of queens and workers was observed by scan sampling using a binocular dissecting microscope. The interval of scans was one to three minutes. Scan sampling was repeated for 30 to 60 minutes. Such observation session was replicated 20 to 60 times. The total observation time was 13 to 48 hours per colony. During the observation, I recorded all behaviours shown by queens, and oviposition and oophagy by workers. Furthermore, to detect egg-laying activity of individual queens and workers, only oviposition and subsequent behaviour was recorded from the observation under a binocular dissecting microscope for 24 hours per colony in colonies A-D. Survival of eggs was estimated from observations of oviposition and the subsequent behaviour of nestmates. After the observation, all queens were dissected under a binocular microscope to check insemination, ovarian development, and yellow bodies as in Ito (1990).

RESULTS

Aggressive interactions and dominance hierarchy among queens, and queen expulsion

Aggressive antennation, biting, and pulling between queens were frequently observed in three functionally monogynous colonies (colonies A, E and F), but it was rare in one functionally monogynous (colony B) and the two monogynous colonies with virgin queens (C and D). These behavioural interactions were similar to those described for workers or intercastes of other species of formicoxenine ants, *Harpagoxenus sublaevis* Nylander, 1849, *Leptothorax allardycei* Mann, 1920, and *Leptothorax* sp. A (Franks & Scovell, 1983; Cole, 1981; Heinze & Smith, 1990).

In colony A, observed just after hibernation, aggressive interactions among queens were frequent, but a clear dominance hierarchy was not found because aggressive interactions were observed only three dyads (Fig. 1). However, one queen started to lay eggs. In colony B, which comprised five mated queens, queen-queen aggressive interactions were quite rare. In this colony, the expulsion of a queen by workers was observed (Fig. 1). On day 3 after the start of observation, the abdomen of one queen (Q3) in colony B became swollen but she did not lay eggs yet. From day 5 to day 6, Q3 was frequently attacked by Q2 and workers in the nest chamber. On day 7, she was always in the foraging arena and received aggressive attacks from workers and Q2 who often engaged in foraging. Q3 did not enter the nest chamber after this day. Two days later, the other queen (Q1) became fertile, having a swollen abdomen, and subsequently started oviposition. After the expulsion of Q3, Q1 laid six eggs, all of which survived. In colonies C and D where there was only one mated queen with some virgin queens, no aggressive

behaviour was observed among queens. In these two colonies, virgin queens frequently showed foraging just like workers.

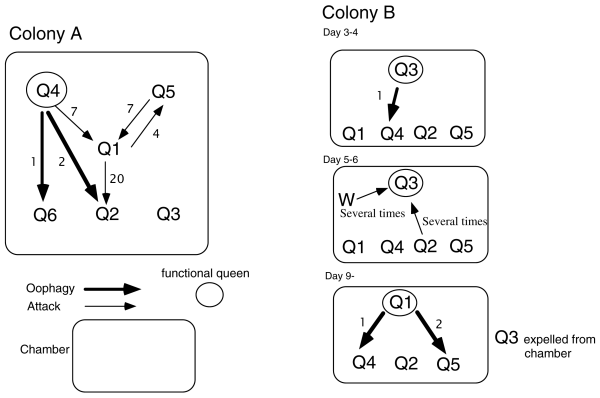

Fig. 1. – Dominance structure in colony A (Q3 was virgin and other queens mated) and successive change of dominance structure in colony B (all queens mated). A numeral on each arrow means the number of observation episodes.

In two colonies (E and F) observed in July, antennation and biting were frequently observed among the supernumerary queens. Top-ranking functional queens never showed such antagonistic behaviour. The supernumerary queens showed remarkable avoidance behaviour against these functional queens : they fled approaching the functional queens. Such avoidance behaviour, only shown against functional queens, was not observed in other colonies. Aggressive interactions including remarkable avoidance behaviour occurred in the same direction in most of a given pair of queens in colony E (Table 2). Therefore, an almost linear dominance hierarchy among dealate queens was established until day 4 (index of linearity, $K' = 0.82$, $P = 0.057$; De Vries, 1995). However, this relationship was not stable : on day 4, Q7 who ranked third, was frequently attacked by workers and Q2. The second ranked queen (Q6) was also attacked by workers. On day 6 after the start of observation, Q6 and Q7 always spent their time in the foraging arena. They often tried to enter the nest chamber; however, they were always attacked by workers. I removed them from the nest and dissected them on day 7 : they had mated but were sterile without yellow bodies. After removal of the two queens, the dominance order was not changed : the fourth ranked queen (Q2) became the second rank, however, she immediately became the target of attacks by workers and Q4. Finally, she was also expelled from the nest chamber on day 9. A similar dominance hierarchy was observed in colony F ($K' = 0.82$, $P = 0.057$). In this colony, the second ranked queen (Q3) was expelled from the nest chamber by intensive attacks from the fifth ranked queen (Q4). After the expelling, the third-ranked queen (Q5) moved to 2nd place.

TABLE 2

Dominance hierarchy and reproductive condition of queens in colonies E and F. Frequencies of aggressive antennation, biting, and pulling were summed. Frequency of remarkable avoidance behaviour was shown in parenthesis.

Colony E	**Subordinate**							
	Q1	**Q6**	**Q7**	**Q2**	**Q4**	**Q5**	**Q3**	**Total**
Dominant								
Q1	---	(3)	(1)	(8)	(4)	(4)	0	(20)
Q6	0	---	15	14	8	5	5	47
Q7	0	0	---	8	4	1	2	15
Q2	0	0	5	---	29	13	5	52
Q4	0	0	0	8	---	4	2	14
Q5	0	0	0	0	0	---	1	1
Q3	0	0	0	0	0	0	---	0
Total	0	(3)	20 (1)	30 (8)	41 (4)	23 (4)	15	129 (20)
Insemination[1]	+	+	+	+	+	-	+	
Ovarian development[2]	+++	-	-	-	-	-	-	

Colony F	**Subordinate**							
	Q6	**Q3**	**Q5**	**Q2**	**Q4**	**Q1**	**Q7**	**Total**
Dominant								
Q6	---	(4)	(3)	(1)	(3)	0	(3)	(14)
Q3	0	---	34	6	28	6	1	75
Q5	0	0	---	21	80	50	1	152
Q2	0	0	0	---	5	2	1	8
Q4	0	10	0	0	---	4	0	14
Q1	0	0	1	1	2	---	4	8
Q7	0	0	0	0	0	0	---	0
Total	0	10 (4)	35 (3)	28 (1)	115 (3)	62	7 (3)	257 (14)
Insemination[1]	+	+	+	+	+	-	+	
Ovarian development[2]	+++	-	-	-	-	-	-	

1.+ mated, - virgin
2.+++ well developed ovaries having many mature oocytes, - undeveloped ovaries

TABLE 3

Egg laying and oophagy in six *L. acervorum* colonies.

Egg layers (No. individuals)	No. eggs laid	No. eggs eaten by				No. eggs survived (%)
		Functional queens	Supernumerary queens	Virgin queens	Workers	
Functional queens (6)	31	0	0	0	0	31 (100)
Supernumerary queens (19)	9	7	0	0	1	1 (10)
Virgin queens (15)	1	0	0	0	1	0 (0)
Workers (211)						
reproductive eggs	17	5	0	0	6	6 (35)
trophic eggs	9	4	0	0	5	0 (0)
Total	67	15	0	0	14	38

Egg cannibalism

Egg cannibalism among nestmates was observed in all six colonies. Since egg-laying activity was low in all colonies, the data were summed (Table 3). During 234 hours of observation for six colonies, oviposition was observed 67 times : 31 by functional queens, 9 by supernumerary queens, one by a virgin queen and 26 by workers. Of 26 eggs laid by workers, 17 were evidently reproductive eggs which were observably indistinguishable from queen eggs in shape, size, and colour, and the remaining nine eggs were trophic eggs which were round because of an undeveloped chorion. All trophic eggs were eaten by functional queens (four eggs) or workers (five eggs). Oophagy of reproductive eggs was observed on 20 occasions, all of which occurred directly after oviposition. In an extreme case, an egg just appeared at the tip of the abdomen of a supernumerary queen was immediately picked up by the consumer. Eggs piled on the nest floor were never destroyed during the observation time. Once an egg was put on the egg-pile, it most likely escaped cannibalism and survived. The survival of reproductive eggs was significantly different among functional queens, supernumerary queens, and workers (Monte Carlo contingency table test (ENGELS, 1988), $P = 0.000 \pm 0.000$SE). The eggs of functional queens were never eaten by nestmates while all but one egg laid by supernumerary queens was immediately eaten by functional queens or workers. Supernumerary queens and virgin queens never showed oophagy. Only one egg laid by a supernumerary queen was intact during three hours after oviposition. In this case, the functional queen licked the egg just after egg laying by the supernumerary queen, but did not attack the egg. Worker-laid eggs also suffered heavy cannibalism by functional queens and workers : only six of 17 reproductive eggs survived.

DISCUSSION

To date, functional monogyny in *L. acervorum* has been reported from Alaska, Spain, and northern Japan (HEINZE & SMITH, 1990; ITO, 1990; FELKE & BUSCHINGER, 1999), while facultative polygyny is known from populations of central, western and northern Europe (BUSCHINGER, 1968; STILLE et al., 1991; BOURKE, 1991; HEINZE et al., 1995). In a Japanese population of *L. acervorum*, aggressive interactions among mated queens were observed as in an Alaskan colony of *L. acervorum* (HEINZE & ORTIUS, 1991) and other functionally monogynous formicoxenine species, *Leptothorax* sp. A and *L. gredleri* (HEINZE & SMITH, 1990; HEINZE et al., 1992). Such queen antagonism is rarely found in European populations of *L. acervorum*. The present study indicates that the geographic difference of behavioural characteristics is an important proximate factor affecting the differences in social structures. Queen antagonism was not found in monogynous colonies with virgin queens. Virgin queens were excluded from reproductive competition among queens in *L. acervorum*, because they functioned just like workers as shown in this paper and in European populations (BUSCHINGER, 1983; BOURKE, 1991).

Even though sample size (number of colonies observed) was small, the frequency of aggressive behaviour among queens was different between two seasons. In spring, the frequency was low but functional queens showed aggressions to others while functional queens never showed actual physical aggression to other queens in summer, even aggression among subordinate queens was frequent. Previous reports on dominance hierarchy in *Leptothorax* ants showed that the aggressive interactions are more frequent after hibernation than in the reproductive season (HEINZE et al., 1992; ORTIUS & HEINZE, 1999), because during the reproductive season queens recognize ovarian development of each other and they rarely attack fertile queens (ORTIUS & HEINZE, 1999). Less aggression against functional queens during the reproductive season is also observed in Japanese *L. acervorum*. As in other species of *Leptothorax* and some other ponerine ants (ORTIUS & HEINZE, 1999; PEETERS et al., 1999; CUVILLIER-HOT et al., 2002), the fertile signal of the alpha individual may be more important than physical direct aggression after establishment of dominance. The reasons of the high frequency of aggressions among supernumerary queens during summer in the Japanese population of *L. acervorum* are unknown in this time.

Among four functionally monogynous colonies, queen expelling was observed in three colonies. In two colonies with a clear dominance hierarchy, second ranked and third ranked supernumerary queens (in total four supernumerary queens) were the target of attacks by lower ranked supernumerary queens and workers. However, it is not sure whether functionally monogynous colonies of *L. acervorum* in northern Japan become truly monogynous via such queen expelling. Queen expelling is also observed in functionally monogynous colonies of *Leptothorax* sp. A, where higher ranked individuals tend to disperse or to be attacked and expelled from colonies (ORTIUS & HEINZE, 1995). In the queenless ponerine ant *Pachycondyla sublaevis* (EMERY, 1877) which shows a

linear dominance hierarchy among workers, and where the top-ranked one is a gamergate (mated and egg-laying worker), HIGASHI et al. (1994) have reported in a colony after artificial removal of the gamergate that the second to fourth ranked workers were aggressively attacked by lower ranked workers, and were expelled from the colony. MONNIN and PEETERS (1999) also reported immobilization and expelling of high ranked workers in the queenless ponerine ant *Dinoponera quadriceps* KEMPF, 1971. Thus, in ant colonies with a linear dominance hierarchy established by aggressive behaviour, expelling higher ranked individuals from colonies may be a common phenomenon. In ant societies where reproduction is regulated by such a dominance hierarchy, low ranked individuals seem to have a lower probability to be a reproductive individual than higher ranked individuals. These observations on ponerine and formicoxenine ants, however, indicate that the higher ranked ones risk to be attacked and expelled from colonies while lower ranked queens can safely stay in the colony and lower ranked queens still may have a probability to become future reproductives. This may be one of the reasons for lower-ranked queens to stay in functionally monogynous colonies.

Queen behaviour in the population of northern Japan is remarkably different from that in the population of central Europe studied by BOURKE (1991). The most striking difference is the occurrence of queen antagonism as mentioned above. In both populations, oophagy was frequently observed; however, behavioural characteristics of oophagy are very different between the European and the Japanese populations. In the European population, the queens ate mostly old eggs piled on the nest floor (BOURKE, 1991) and freshly-laid eggs were rarely eaten by queens who showed random egg eating without discrimination between nest mate eggs and their own eggs (BOURKE, 1994). In the case of the Japanese population, oophagy always occurred directly after oviposition and eggs piled on the nest floor were never eaten. Queens and workers ate eggs preferentially. The frequency of oophagy was also greatly different : in the European population, the number of eggs eaten / number of eggs laid during observation was 0.75 (calculated from BOURKE (1991)) while in the Japanese population it was just 0.22. Furthermore, workers also showed oophagy of reproductive eggs laid by supernumerary queens in the Japanese population while this was rare in the European population. The remarkable behavioural differences in oophagy seem to be a consequence of the difference in reproductive structure : strong selection for functional monogyny may lead to preferential oophagy by functional queens and workers in Japanese colonies. As shown by BOURKE (1994), queens of *L. acervorum* could not discriminate own eggs from the other's eggs. Thus, selection would favor oophagy directly after oviposition when the egg-layer could be identified. Such oophagy directly after oviposition is also shown in the ponerine ants *Diacamma* sp., *Pachycondyla villosa* Fabricius, 1804, and *Dinoponera quadriceps,* and the myrmicine ant *Acanthomyrmex ferox* Emery, 1893 (PEETERS & TSUJI, 1993; HEINZE, et al., 1996, MONNIN & PEETERS, 1997; GOBIN & ITO, 2000).

Dissection of *L. acervorum* queens collected in the field (ITO, 1990) and behavioural observations shown in this paper indicated that some supernumerary queens laid eggs at a low rate under the presence of a functional queen. The results shown here suggest that these eggs could not survive under the occurrence of preferential egg cannibalism by functional queens and workers. Therefore, functional monogyny in Japanese *L. acervorum* is completed by preferential egg cannibalism in addition to queen antagonism.

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Keratinization in crocodilian scales and avian epidermis : evolutionary implications for the origin of avian apteric epidermis

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ABSTRACT. Terminal differentiation of keratinocytes of avian apteric epidermis occurs with the accumulation of little keratin and much lipids so that a soft and elastic corneous layer is produced. The distribution of keratins and some proteins associated with cornification has been studied in crocodilian scales, in ratite and zebrafinch apteric epidermis by means of light and ultrastructural immunocytochemistry. Soft (alpha)keratinization in apteric epidermis of birds resembled the process occurring in hinge regions of crocodilian scales where the stratum corneum was thin and beta(hard)-keratin disappeared. Acidic and basic alpha-keratins were seen in living pre-corneous layers. Instead, keratins typical for cornification, loricrine, transglutaminase, and sometimes filaggrin-like immunoreactivities, were present in the transitional and lowermost corneous layer of crocodilian hinge and apteric avian epidermis. Trichohyalin, involucrin, and iso-peptide bond immunoreactivities were absent. Loricrine-like and transglutaminase labelling were generally absent in the corneous layer, but were weakly present among keratin bundles and lipids in the transitional layer of apteric epidermis. Transglutaminase immunolabelling was present in condensing nuclear chromatin of transitional corneocytes of apteric epidermis, suggesting that these cells undergo terminal differentiation or even apoptosis. Sulfydryl groups in keratins, or specific sulfur-rich proteins of loricrin-type, were scarce in apteric epidermis. This suggests that the cornified cell envelope of avian keratinocytes is more simplified than that of mammalian keratinocytes. In the latter, numerous proteins concentrate along the corneous cell envelope to enhance mechanical and chemical resistance of the stratum corneum. This mechanism probably is more simplified in apteric epidermis of birds, and the mechanical protection of the epidermis is taken over by the plumage, while apteric epidermis has mainly a role as barrier against water loss. It is speculated that avian interfollicular and apteric epidermis has evolved from interscale hinge regions of proto-avian archosaurian ancestors.

KEY WORDS : crocodilians, ratite birds, epidermis, keratinization, immunocytochemistry.

INTRODUCTION

From the hard, scaled epidermis of archosaurian, and perhaps of theropod reptiles, scales and feathers in birds have probably evolved (SPEARMAN, 1966; MADERSON, 1972; BRUSH & WYLD, 1980; BRUSH, 1993; CHUONG et al., 2000; PRUM, 2002). It is thought that the evolution of the avian integument from scaled archosaurian reptile ancestors (MADERSON, 1972; MADERSON & ALIBARDI, 2000) has originated a soft epidermis (apterilae) among feathers (pterylae), while scales have remained in the skin of the hindlimb (ALIBARDI, 2003a, b, 2004a, b).

Previous morphological and molecular studies (SPEARMAN, 1966; MADERSON, 1972; GREGG & ROGERS, 1986; SAWYER et al., 1986; SAWYER et al., 2000; MADERSON & ALIBARDI, 2000), together with recent embryological, ultrastructural and immunocytochemical evidence (ALIBARDI & THOMPSON, 2000, 2001; ALIBARDI, 2003a, 2004b), suggest that the expansion of the hinge regions among scales of the proto-avian archosaurians might have originated the apteric epidermis. This pliable and elastic epidermis has transformed the rigid and scaled reptilian armour, into a flexible and articulate skin. While plumage and scales are epidermal appendages specialized for flying and mechanical protection, the lipid-rich and delicate apteric skin hosts the permeability barrier against water loss (MENON et al., 1986; MENON et al., 1996; MENON & MENON, 2000). The apteric and interfollicular epidermis also gives the mechanical plasticity necessary for feather orientation during flight (HOMBERGER & DE SILVA, 2000), participates in the homeothermic regulation by heat dissipation, secretes specific lipid-rich materials, and hosts special glands (LUCAS & STETTENHEIM, 1972; SPEARMAN & HARDY, 1985).

Presently, much information is available on the ultrastructure, biochemistry and molecular biology of feather and scale epidermis (MATULIONIS, 1970; BRUSH & WYLD, 1980; GREGG & ROGERS, 1986; SAWYER et al., 1986; 2000; CHUONG, 1993; CHUONG et al., 2000; ALIBARDI, 2002a). As opposed, except from the ultrastructure and lipid composition (LAVKER, 1975; ELIAS et al., 1987; SPEARMAN & HARDY, 1985; MENON et al., 1986, 1996; PELTONEN et al., 1998, 2000; MENON & MENON, 2000), little is known on the expression of specific proteins during the terminal differentiation of keratinocytes in apteric epidermis.

In mammalian epidermis, the synthesis of different proteins (involucrin, loricrin, filaggrin etc.) from the upper spinosus or in the granular layer, determines the formation of the transitional and corneous layer (FUCHS,

1990; RESING & DALE, 1991; RAWLINGS et al., 1994; KALININ et al., 2002; ALIBARDI & MADERSON, 2003). The production of similar proteins during maturation of keratinocytes in apteric epidermis of birds is not well known although new information is emerging (ALIBARDI, 2004a, b, ALIBARDI & TONI, unpublished observations). To address some of these questions, an ultrastructural and immunocytochemical study has been done on the distribution of keratins, loricrin, filaggrin, transglutaminase and isopeptide-bond in apteric epidermis of ratite birds, in comparison with the epidermis of crocodilian scales. Such a comparison aims to check whether the characteristics of keratinocytes of crocodilian hinge regions resemble those of keratinocytes of apteric epidermis in birds, termed sebokeratinocytes (MENON & MENON, 2000). This further supports the hypothesis that apteric epidermis is derived from hinge regions of proto-avian archosaurs (ALIBARDI, 2003a).

MATERIAL AND METHODS

Two young Australian saltwater crocodile, *Crocodyle porosus*, and 2 late embryos of the American alligator, *Alligator mississippiensis*, were used in the present study. The skin was collected from biopsies of the neck, belly, lateral mid body (softer scales), and from back and tail scales (harder scales), as previously detailed (ALIBARDI & THOMPSON, 2000; ALIBARDI, 2003a). Tissues (2-4 mm in length) were fixed for 5-8 hours in Carnoy fluid (9 parts 80% ethanol + 1 part acetic acid), dehydrated, and embedded in Lowcryl K4M resin under UV for 2-3 days at 0-4°C. Other tissues were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 post-fixed in osmium, dehydrated, and embedded in Durcupan resin.

Two zebrafinch embryos, from 12 days post-deposition eggs, were fixed in 2.5% glutaraldehyde as above. Other four zebrafinches were injected in the abdomen with of a Ringer solution containing tritiated histidine (L 2,5 3H-histidine, specific activity 40-60 Ci/mole, Amersham, UK) at a dosage of 2-4 μCi/g body weight. Samples of skin from the ventral and pectoral regions were collected 4 hours after the injection, for the histoautoradiographic study on skin sections.

From four adult emus (*Dromaius novae-hollandiae*) little pieces (3-5 mm) of skin were collected from a featherless area about 12 cm above the wing toward to the neck, and immediately fixed. Some tissues were fixed in 10% formaldehyde for 5-6 hours, stored in 0.1 M phosphate buffer for a few days, post-fixed in 2.5% glutaraldehyde for 2 hours, then in 2% osmium for 2 hours, dehydrated and embedded in Spurr resin. Other tissues were fixed in Carnoy's fluid, dehydrated, and embedded in Lowcryl K4M resin under UV-light. Fresh skin samples were obtained from the head and neck of four ostriches (*Struthio camelus*). Some tissues were fixed in 2.5% glutaraldehyde as above, post-fixed in osmium, dehydrated and embedded in Durcupan resin. Other pieces were fixed in 3% paraformaldehyde in phosphate buffer or in Carnoy's for 4-6 hours, and then embedded in Lowcryl K4M resin as above.

Semithin sections of the embedded tissues (1-4 μm thick) were obtained using a LKB-Nova ultramicrotome, and attached to gelatin-coated slides for the following immunocytochemical stain. From some areas of the skin, thin sections (40-90 nm thick) were collected on copper or nickel grids, stained with uranyl acetate and lead citrate, and observed under a CM-100 Philips electron microscope.

For autoradiography, tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for about six hours, post-fixed in 1% osmium tetroxide for one hour, dehydrated and embedded in the resin Durcupan. Sections of 2-4 μm thickness were coated with Ilford K5 Nuclear Emulsion, exposed in a darkroom for 1-2 months, developed in Kodak D19 and fixed in Ilfor fixer. Sections, either unstained or lightly stained with 1% toluidine blue, were studied under the light microscope for detection of the silver grains derived from the autoradiographic process. Other thin sections (40-90 nm thick) were collected onto collodion-coated slides and coated with Ilford L4 Nuclear Emulsion for ultrastructural autoradiography. After 3-4 months exposure, sections were developed as above, collected on nickel grids, stained and observed under the electron microscope.

For light microscopic immunocytochemistry, anti-keratin mammalian antibodies (AE1, AE2, AE3) were purchased from Progen (Heidelberg, Germany). They recognize most acidic and basic alpha-keratins (SUN et al., 1983; O'GUIN et al., 1987). The anti-filaggrin antibody was produced and generously supplied by Dr. BA Dale (Department of Oral Biology, University of Washington, Seattle, USA). It is a polyclonal antibody (#466) produced in rabbit and directed against rat filaggrin in both sections and blottings. The anti-loricrin antibody, produced in rabbit, was generously supplied by Dr. E. Fuchs (Howard Hughes Medical Institute, University of Chicago, USA), and recognizes a 15 amino acidic sequence toward the C-terminal of mouse loricrin (MEHREL et al., 1990). The antitrichohyalin antibody was kindly supplied by Dr. G. Rogers (University of Adelaide, Australia), and recognizes trichohyalin from sheep, rat and other mammals (ROTHNAL & ROGERS, 1996; A ALIBARDI, personal observations). The anti-involucrin antibody is a mouse monoclonal antibody directed against human involucrin (I-9018, Sigma, USA). The antibody ab421 against transglutaminase 1 of guinea pig liver (rabbit polyclonal), and the ab422 against N-?-(?-glutamyl)-lysine isopeptide bond (isopetide-bond, mouse monoclonal) were purchased from Abcam Limited, Cambridge, UK. Finally, the beta-1 is a rabbit polyclonal antibody directed against a chicken scale beta-keratin, and was generously supplied by Dr. R. Sawyer (University of South Carolina, Columbia, USA; SAWYER et al., 2000).

Tissues were preincubated for 30 minutes in 5% normal goat serum in 2% BSA in 0.05 M Tris/HCl buffer at pH 7.6, incubated overnight at 4° C in the buffer containing the primary antibody (dilutions, 1 : 50-100 for alpha-keratin antibodies, 1 : 500 for filaggrin, 1 : 200-300 for loricrin, 1 : 150 for transglutaminase, 1 : 150 for iso-peptide, 1 : 500 for trichohyalin, 1 :200 for beta-1 keratin, and 1 : 100 for involucrin). After several rinses in buffer, the sections were incubated in the same medium for one hour at room temperature containing 1 : 50 of anti-mouse-IgG (for antikeratin, involucrin, and iso-peptide)

or anti-rabbit-IgG (for filaggrin, loricrin, beta-1, transglutaminase, and trichohyalin) -FITC conjugated secondary antibodies. After rinsing, sections were mounted in Fluoromount (EM Sciences, USA), and observed under a Zeiss epifluorescence microscope equipped with a fluorescein filter.

For immunoelectron microscopy, 40-90 nm thick sections were collected on nickel grids, and immunostained with the primary antibody against loricrine and transglutaminase as above (in controls the primary antibody was omitted). As secondary antibody an anti-mouse or anti-rabbit IgG conjugated to 10 nm large gold particles were used (Sigma, USA, or Biocell, UK). Sections were observed under the electron microscope either unstained or lightly stained with uranyl acetate, under a CM-100 Philips electron microscope operating at 60-80 kV.

RESULTS

Crocodilian scales

Crocodilian scales formed from narrow hinge regions among expanding outer scale surfaces (Fig. 1 A). By the end of development, as well as in adult scales, hinge regions remained as narrow areas between the outer surface of scales (Fig. 1 B). The outer scale surface will progressively expand into larger scales during the growth of the animal.

The epidermis of the young crocodilian scales consisted in a basal layer made of polygonal cells, 3-6 suprabasal layers made of flat cells, a transitional pre-corneous layer, a variably thick stratum corneum (for details see ALIBARDI, 2003a). Most of epidermal surface was formed by the dorsal part of scales which were delimited by narrow hinge regions. The corneous layer of alligator and crocodilian scales appeared thinner in hinge regions, where a higher AE2-immunoreactivity for alpha-keratin was present (Figs 1 C-D). As opposed, the immunoreactivity of the thicker epidermis of the dorsal portion of scales was immuno-negative for AE2-alpha-keratin, but immunopositive for beta-1 keratin which tended to disappear in the hinge regions (Figs 1 D-E). Corneocytes (mature keratinocyets of the stratum corneum) in the dorsal part of the scale were 0.5 μm or thicker, and featured a spiny or tortuous surface (Fig. 1 F). Bundles of beta-keratin accumulated onto the plasma membrane in the transitional layer. The mature cells of the beta-keratin corneous layer consisted in compact corneocytes. Melanosomes were mixed to beta-keratin, especially in dorsal scales in both alligator and crocodile.

In the hinge region, suprabasal keratinocyets accumulated numerous lipid vesicles and smooth endoplasmic reticulum, mitochondria often showed tubular cristae and tonofilaments were sparse (Fig. 1 G). In pre-corneous keratinocytes bundles of tonofilaments tended to accumulate along the thickened plasma membrane, and the core contained lipids and dense, mucus-like, granules (Fig. 1 H). Occasional beta-keratin bundles were present in these cells.

Corneocytes of hinge regions were very narrow (0.05-0.2 μm), and showed a smooth surface (inset in Fig. 1 H). The latter morphology resembled that of sebokeratinocytes in avian apteric epidermis (see later).

Apteric epidermis

In the zebrafinch embryo few large apteric regions formed between pterylae tracts (Fig. 2 A). The epidermis of ratites and zebrafinch was very similar, although variations in thickness were seen (Figs 2 B-C). Above a cubic to flat basal layer, 2-4 layers of flat intermediate cells preceded the straum corneum, which was made by several layers of thin cells. The basement membrane followed the tortuous indentation sometimes present along the basal cytoplasm of germinal cells. The latter, among the usual organelles, contained sparse bundles of tonofilaments which increased in diameter, density and dimension in suprabasal and pre-keratinized cells of the transitional layer (Figs 2 D-E). Bundles of electron-dense alpha-keratin were accumulated along the plasma membrane. The transitional or pre-corneous layer marked the passage from upper intermediate cells to completely keratinized cells of the stratum corneum (sebokeratinocytes). No keratohyalin granules could be differentiated from the irregularly sectioned tonofilament bundles that were present in transitional cells. Lipid vacuoles tended to concentrate in the middle of transitional and corneous cells, the plasmalemma became thicker and electrondense forming a cornified cell envelope (Figs 2 D-E). Lamellar figures were often seen extracellularly among corneous cells.

In the epidermis of the zebrafinch, 4 hours after tritiated histidine injection, the labelling appeared evenly distributed over all layers of the epidermis, and absent in the dermis (Fig. 2 F). The ultrastructural details of the labelling showed that silver grains were generally associated with the diffuse or condensed keratin filaments of transitional or mature sebokeratinocytes (Figs 2 G-H).

The AE1 immunofluorescence was mainly or exclusively seen in the germinal and first suprabasal layers of ratite epidermis (Figs 2 I-J), the AE2 antibody mainly stained the corneous and transitional layers (Figs 2 K-I, 3 A). Finally, the AE3 stained mostly living layers while the corneous layer (made of sebokeratinocytes) was generally less stained or not stained at all (Figs 3 B-C). The AE2 antibody sometimes also produced a nuclear staining, which was seen in many but not all nuclei of ratite epidermis.

In some apteric areas the anti-filaggrin antibody reacted over the transitional layer, while a large part of the corneous layer remained unstained (Figs 3 D-E). The anti-loricrin antibody produced a stronger and more consistent reaction than that against filaggrin, and the staining was more evenly distributed over all areas of apteric epidermis, especially in the transitional and lowermost part of the corneous layer (Figs 3 F-H). The transglutaminase antibody immunostained the transitional and lowermost corneous layer of the epidermis, but the immunofluorescence was patchy or completely absent in the corneous layer (Fig. 3 I). No immunolabelling was observed using antibodies directed against iso-peptide, trichohyalin and involucrin.

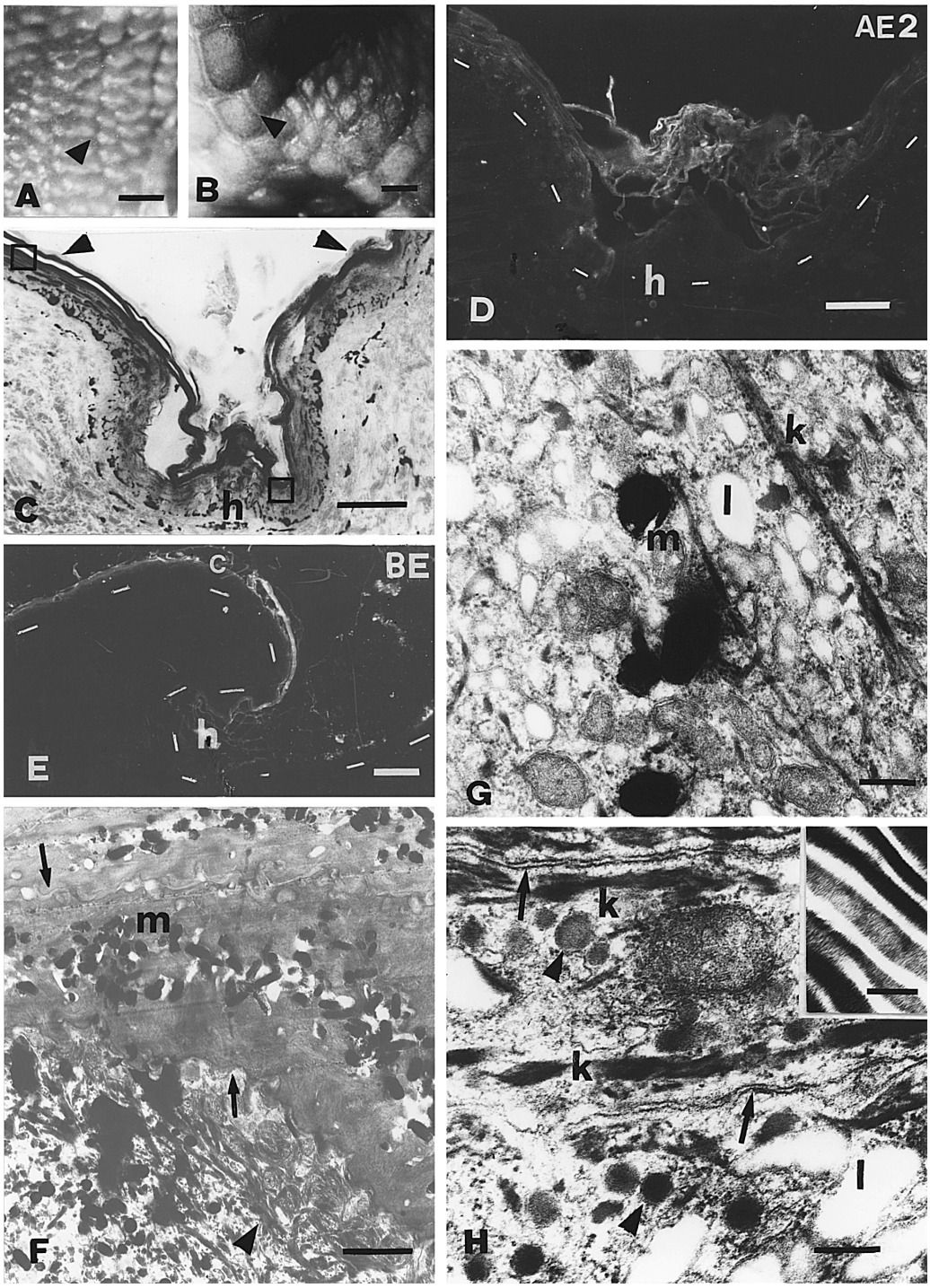

Fig. 1. – **A**, latero-ventral forming hinge regions (arrowhead) among the outer scale surface of scales of late alligator embryo. Bar, 0.5 mm. **B**, narrow hinge regions (arrowhead) among neck scales of late alligator embryo. Bar, 0.5 mm. **C**, hinge region of dorsal scale of alligator showing the disappearence of the pale beta-layer of the stratum corneum of scales (arrowheads). Bar, 40 µm. **D**, AE2-immunopositive corneous layer in hinge region of alligator dorsal scale. Bar, 30 µm. **E**, Beta-1 immunopositive corneous layer of outer surface of alligator dorsal scale, which disappears in the hinge region. Bar, 50 µm. **F**, accumulation of beta-keratin bundles (arrowhead) in pre-corneous cells of neck scale in crocodile. Numerous melanosomes are accumulated inside mature corneocytes which show an irregular surface (arrows). Bar, 1 µm. **G**, cytoplasm of differentiating keratinocyte in the hinge region of alligator dorsal scale where numerous lipid vesicles and sparce keratin bundles and mitochondria are seen. Bar, 0.25 µm. **H**, pre-corneous keratinocyte in the hinge region of alligator dorsal scale showing parallel alpha-keratin bundles, lipid vesicles, mucous-like granules (arrowheads), and thickened plasma membrane (arrows). Bar, 0.25 µm. Inset, narrow corneocytes in hinge region of alligator scale. Bar, 0.2 µm. **Legends** : AE2, AE2-immunoreaction for alpha-keratins; BE, beta-1 immunoreaction for beta-keratin; c, corneous layer; h, hinge region; k, keratin bundles; l, lipid material. m, melanosomes. Dashes underline the basal layer.

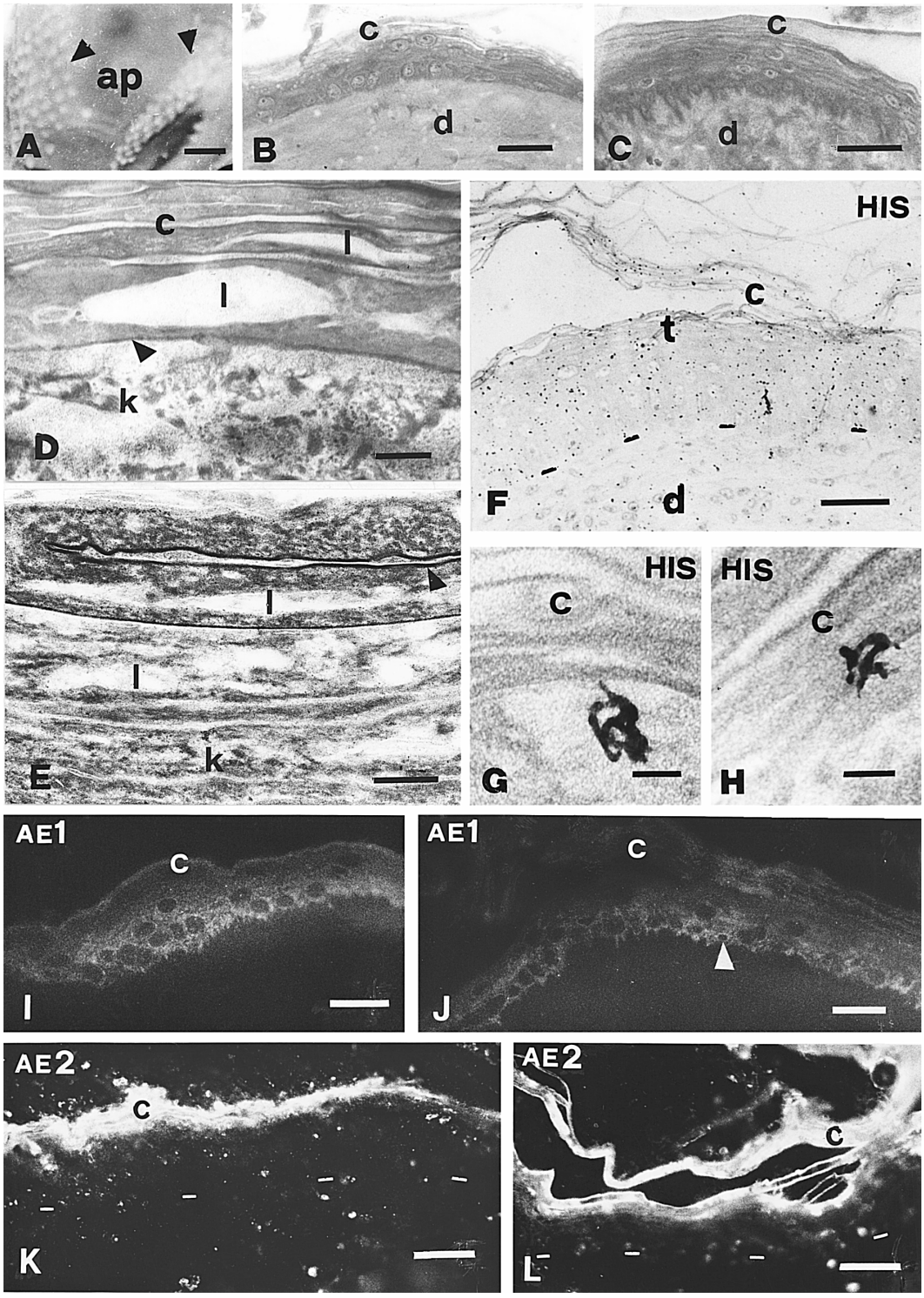

Fig. 2. – **A**, overview of zebrafinch embryo showing the smooth, apteric region between rows of feather germs (arrowheads) in the lumbar region. Bar, 1.5 mm. **B**, thin epidermis of emu. Bar, 20 µm. **C**, epidermis of ostrich. Bar, 20 µm. **D**, sparce keratin bundles and dense cornified cell envelope (arrowhead) in cell of the transitional layer of emu epidermis. Bar, 0.25 µm. **E**, merging lipid vacuoles among keratin bundles in cells of the transitional layer of ostrich epidermis (arrowhead on cornified cell envelope). Bar, 0.25 µm. **F**, evenly labeled zebrafinch epidermis 4 hours after tritiated histidine injection. Bar 15 µm. **G**, ultrastructural detail of autoradiographic silver grain over pale cytoplasm of transitional cell in zebrafinch epidermis. Bar, 0.2 µm. **H**, ultrastructural detail of autoradiographic silver grain over keratin filaments in sebokeratinocytes. Bar, 0.2 µm. **I**, AE1 immunofluorescence in emu epidermis. Bar, 15 µm. **J**, AE1 immunofluorescence in ostrich epidermis (arrowhead on the basal layer). Bar, 15 µm. **K**, AE2 immunofluorescence in emu epidermis. Bar, 20 µm. **L**, AE2 immunofluorescence of ostrich epidermis. Bar, 20 µm. **Legends** : ap, apteric region; c, corneous layer; d, dermis; k, keratin bundles; l, lipid material. Dashes underline the basal layer.

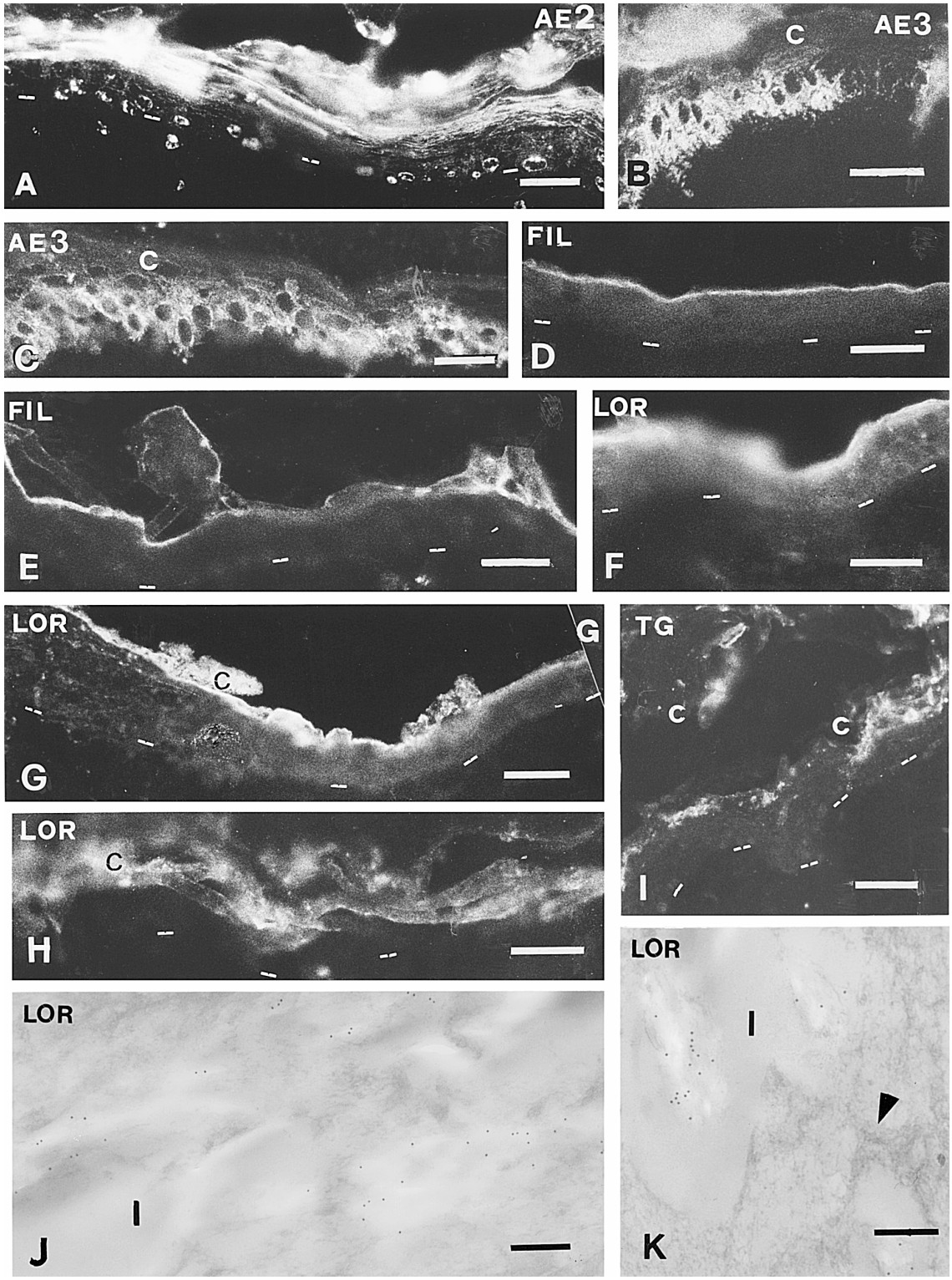

Fig. 3. – **A**, AE2 positive thick corneous layer of ostrich epidermis. Bar, 10 µm. **B**, AE3 immunofluorescence in emu epidermis. Bar, 15 µm. **C**, AE3 fluorescence in emu epidermis. Bar, 15 µm. **D**, thin filaggrin-like immunofluorescent transitional layer of emu. Bar, 20 µm. **E**, filaggrin-like immunofluorescent transitional layer of ostrich. Bar, 20 µm. **F**, loricrine immunoreactive transitional and corneous layer of ostrich. Bar, 20 µm. **G**, loricrine immunoreactive transitional and corneous layer of emu. Bar, 20 µm. **H**, thick loricrine immunoreactive corneous layer of ostrich. Bar, 20 µm. **I**, tranglutaminase immunofluorescence in the transitional and patchy corneous layer (here artefactually fragmented during sectioning). Bar, 20 µm. **J**, loricrine gold immunolabeled pale vesicles within upper spinosus cell of emu epidermis. Bar, 0.25 µm. **K**, loricrine gold labelling within pale vesicle of upper spinosus cell in ostrich epidermis. The arrowhead points to keratin filaments. Bar, 0.25 µm. **Legends** : c, corneous layer; k, keratin bundles; l, lipid material. Dashes underline the basal layer.

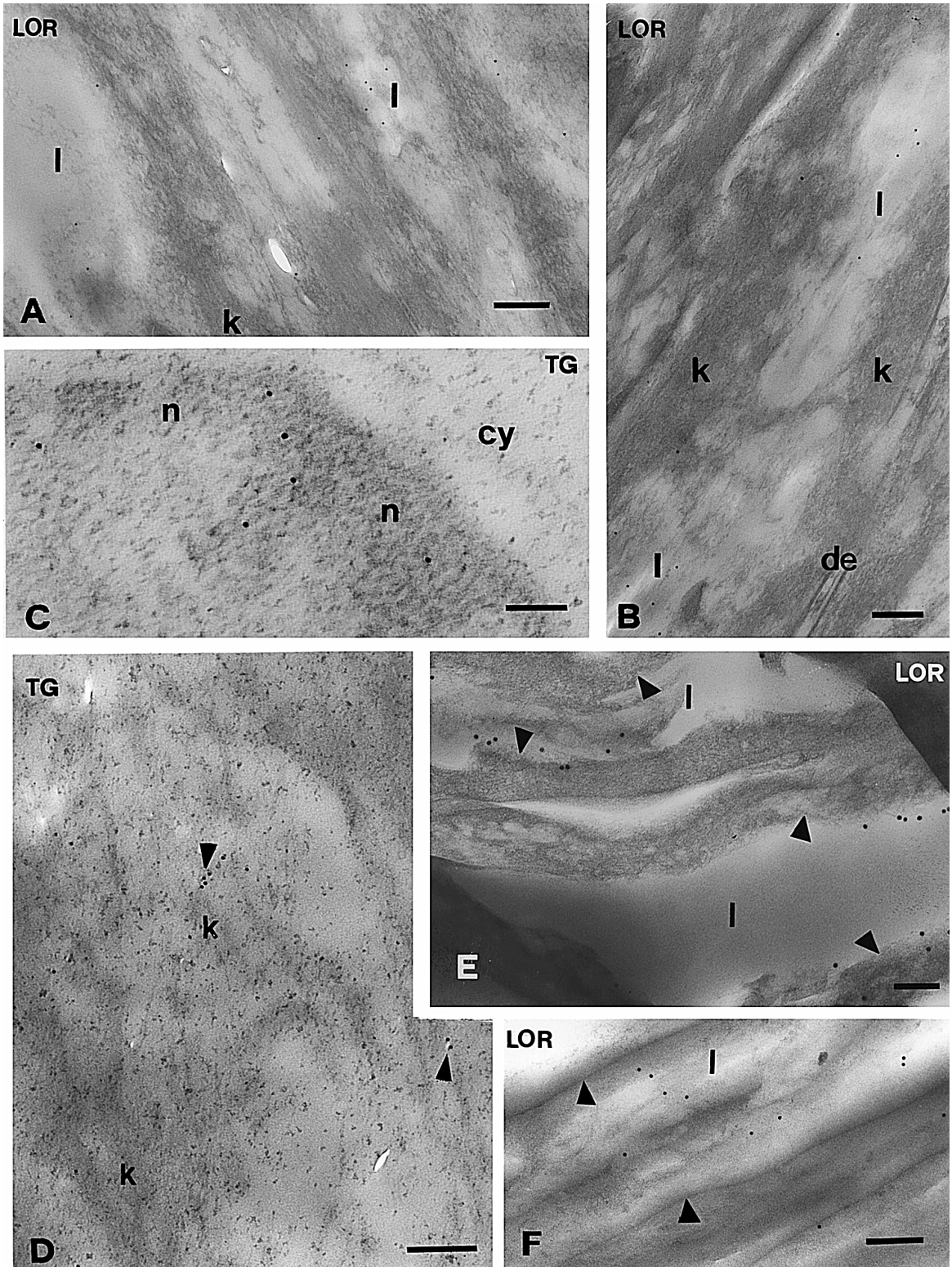

Fig. 4. – **A**, diffuse loricrine immunolabelling among keratin bundles of upper intermediate cell of ostrich. Bar, 0.25 µm. **B**, weak loricrine labelling within the central, lipidic-like spaces of transitional cell of emu epidermis. Bar, 0.25 µm. **C**, transglutaminase labeled nuclear chromatin of upper spinosus cell of ostrich epidermis. Bar, 0.1 µm. **D**, diffuse transglutaminase labelling (arrowheads) among keratin filaments in upper intermediate cell of ostrich epidermis. Bar, 0.2 µm. **E**, loricrine labelling along the external keratin bundles (arrowheads) within sebokeratinocytes of ostrich stratum corneum. Bar, 0.1 µm. **F**, diffuse loricrine immunolabelling within ostrich sebokeratinocytes but not along the cornified cell envelope (arrowheads). Bar, 0.1 µm. **Legends** : c, corneous layer; cy, cytoplasm; de, desmosomes; k, keratin bundles; l, lipid material. n, nucleus. Dashes underline the basal layer.

The ultrastructural localization for loricrin immunoreactivity showed a light but specific labelling associated with lipid-like or pale vesicles in cells of the upper spinosus or in the transitional layer (Figs 3 J-K). A diffuse labelling was seen among keratin bundles or in the core of the flattening sebokeratinocytes among the central lipid material (Figs 4 A-B). The transglutaminase labelling was similar to that for loricrine but present only in transitional cells and more diffuse than the loricrin labelling. It was diffuse over lipid or vacuoles among keratin filaments in transitional cells, and some weak labelling was seen in the condensing perinuclear chromatin of nuclei in upper intermediate cells and in transitional cells (Figs 4 C-D).

The immunolabelling for loricrin in transitional cells contacting mature sebokeratinocytes, was most frequently seen than that for transglutaminase, especially within the central lipid core, but not in the peripheral keratin material beneath the plasmalemma (Fig. 4 E). The immunolabelling was however diffuse or completely absent in most areas of the stratum corneum. Finally, the patchy labelling observed in mature sebokeratinocytes was generally absent along the plasma membrane (Fig. 4 F).

DISCUSSION

Keratins

The present study confirms that distribution of beta-keratin is found only in the dorsal surface of crocodilian scales and is absent in apteric epidermis of ratite, as in the remaining neognate birds (SAWYER et al., 2000; ALIBARDI & SAWYER, 2002; ALIBARDI, 2003a, 2004a). This type of keratin (beta) forms the hard epidermis of reptilian and scutate-scutellate scales of birds.

The present study also shows that alpha-keratin bundles are present in the germinative and intermediate epidermal cells, and include both acidic and basic keratins. The pattern of immunolocalization therefore resembles that of the other amniotes, with acidic and basic keratins associated in most epidermal layers but decreasing in the corneous layer where their reactive epitopes becomes masked or modified (SUN et al., 1983). Mature keratinocytes of avian epidermis, termed sebokeratinocytes, resemble mesos or thin alpha-cells of reptilian epidermis (MENON & MENON, 2000). These lipid-rich cells do not show an alpha-keratin patter under electron microscopic analysis (MATOLTSY, 1969; MADERSON et al., 1972; MADERSON, 1985; LANDMANN, 1979, 1980). This has been correlated with the absence or with the extremely reduced amount of interkeratin (matrix) material present in sebokeratinocytes.

The AE1 immunolabeling, limited to the basal layer in human epidermis (SUN et al., 1983), is less precisely localized in that of crocodilian and ratite epidermis, where not only the basal layer but also some suprabasal cells are labeled, although less intensely (ALIBARDI & THOMPSON, 2002; ALIBARDI, 2003a). Acidic keratins (AE1-positive) are also present in the intermediate and transitional layer of crocodilian and avian epidermis, where they are coupled to basic keratins according to the pair-rule (one acidic keratin molecule is bound to one basic keratin molecule, see SUN et al., 1983; O'GUIN et al., 1987).

The immunocytochemical results suggest that reactive epitopes of acidic keratin becomes somehow masked in the uppermost epidermal layers. The AE2 immunolabelling (for 56.5 and 66-67 kDa mammalian keratins) often shows a similar pattern as that seen using antibodies against filaggrin. It is however known that the AE2 antibody can also cross-reacts with common epitopes between keratins and filaggrin (DALE & SUN, 1983), and therefore the presence of filaggrin-like proteins in avian epidermis requires a biochemical study. The immunolabelling with AE2 in apteric sebokeratinocytes indicates the presence of some high molecular weight keratins involved in cornification (K1, 66-67 kDa and K10, 56.5 kDa; O'GUIN et al., 1987). Basic keratins (AE3 positive) in ratite epidermis appear more abundant, and present in transitional and also the corneous layer (O' GUIN et al., 1987; ALIBARDI, 2004a, b). The AE1, AE2 and AE3 immunoreactive patterns observed in crocodile and ratite epidermis are in general similar to that of the epidermis of other amniotes (SUN et al., 1983; O'GUIN et al., 1987; ALIBARDI, 2002b, c, d, e, 2003a).

The AE2 nuclear staining seen over many but not all of the nuclei in some sections, appears either unspecific or may be due to the recognition of this antibody to nuclear intermediate filaments other then keratin, but with a common antigenic determinant (PRUSS et al., 1981).

Keratin-associated matrix proteins

The dorsal surface of crocodilian scales, which represents most of epidermal surface, is made of relatively larger corneocytes with a rough or spiny surface, and which contain beta-keratin (MADERSON, 1985; SAWYER et al., 2000; ALIBARDI & THOMPSON, 2002; ALIBARDI, 2003a). As opposed, in the hinge regions corneocytes are very narrow, have a smooth surface, contain little or no beta-keratin, but instead lipids, and sparse tonofilaments bundles with prevalent parallel orientation with that of the epidermis. Keratohyalin granules are also absent in these cells in crocodilians, and this condition resembles that present in apteric epidermis of birds.

As in zebrafinch and chick epidermis, filaggrin-like immunoreactivity is weak and uneven over ratite sebokeratinocytes (ALIBARDI, 2004a). This result further suggests that, aside the poor cross-reactivity of this antibody with heterologous species (RESING & DALE, 1991), not much interkeratin (matrix) molecules rich in histidine are present in avian sebokeratinocytes.

The low labelling in the transitional layer of the zebrafinch 4-24 hours after injection of tritiated histidine has indicated that no keratohyaline or histidine-rich molecules are present among keratin bundles of sebokeratinocytes. The filament bundles present in transitional cells of avian epidermis previously indicated as avian keratohyalin (MATOLTSY, 1969; SPEARMAN & HARDY, 1985; PELTONEN et al., 1998, 2000), are not equivalent to mammalian keratohyalin. In fact, these bundles incorporate little or no tritiated histidine, and therefore very little histidine-rich and other proteins are present in these cells.

In conclusion, the present observations suggest that no or little matrix material is present in sebokeratinocytes of

apteric epidermis, as in reptilian mesos and alpha-cells (LANDMANN, 1979, 1980), and in hinge regions among reptilian and avian scales. The negative results using antibodies against trichohyalin, iso-peptide bonds, and involucrin indicate that these proteins are species-specific, and that no cross-reactivity between avian and mammalian epidermal proteins is possible after fixation of cells.

Cornified cell envelope proteins

A very weak loricrine-immunoreactivity is present in hinge regions of crocodilian epidermis (ALIBARDI, 2003a). X-ray microanalysis of the corneous and transitional layer of the chick have indicated the presence of sulfur (ALIBARDI & KAPLIN, unpublished observations) but not of phosphorous (the latter is typical in mammalian keratohyalin; see RESING & DALE, 1991). This may explain the detection of a weak loricrin-like immunoreactivity in the transitional and corneous layers of avian epidermis, as loricrin in mammalian epidermis is a sulfur-rich protein (MEHREL et al., 1990; LEAPMAN et al., 1997). A sulfur-containing protein with some immunocross-reactivity with mammalian loricrin seems to be present in small amount in avian sebokeratinocytes, although it is not concentrated along the cornified cell envelope.

The low amount of both keratin bundles and loricrin-like molecules in avian alpha-layer is confirmed by the poor reactivity to sulfydryl groups (WESSELS, 1961; CANE & SPEARMAN, 1964; SPEARMAN, 1966). Loricrin-like molecules are sparse among lipids and keratin bundles, and the immunolabelling is not localized into specifc granules (L-granules) as in corneocytes of mammals (STEVEN et al., 1990; LEAPMAN et al., 1997; HARDMANN et al., 1998; ISHIDA-YAMAMOTO et al., 2000). Also in human corneocytes a large amount of loricrin is initially distributed within the cytoplasm, but it concentrates along the cornified cell envelope at maturation (ISHIDA-YAMAMOTO et al., 2000). This is not seen in avian, lizard, snake, turtle or in corneocytes of monotremes (ALIBARDI, 2002b, c, d, e; ALIBARDI & MADERSON, 2003), where loricrin-like labelling remains cytoplasmic. This suggests that the antibody does not cross-react with reptilian-avian sulfur-rich proteins after fixation, or that these proteins are under the detection sensitivity of the employed immunocytochemical procedures.

It may also be possible that other proteins more than loricrin form the softer cornified cell envelope of avian sebokeratinocytes, but further biochemical studies are needed. The presence of transglutaminase in the same areas where loricrin-like immunoreactivity is present (loricrin is one of the main substrates of transglutaminase) further suggests that the two proteins are functionally linked during cornification of avian epidermis. The lack of isopeptide-bond detection (which derives from the action of transglutaminase on protein substrate such as loricrin) indicates that so little cross-linking is present in the apteric sebokeratinocytes to be under the detection or accessibility of antibodies.

Transglutaminase appears linked to condensing nuclear chromatine, as it is typical of nuclear membrane cross-linking during apoptosis in mammalian epidermis (HAAKE & POLAKOWSKA, 1993). During terminal differentiation in the transitional layer, nuclei become pycnotic, and are incorporated among the mature corneous layer (MENON & MENON, 2000, present observations). Transglutaminase labelling appears in the condensed perinuclear chromatin already in upper intermediate and precorneous, cells of the epidermis. Keratinization of apteric epidermis is, like that of mammals, probably an apoptotic-driven process.

Avian soft keratinization and evolutive speculations

The present observations in ratite birds confirm the immunolocalization of keratins, associated proteins, and tritiated histidine in the epidermis of neognates birds (ALIBARDI, 2003b, 2004a, b). These results probably indicate that the pattern of keratinization of apteric epidermis is similar in all extant birds. Complex lipids and waxes are synthesized in sebokeratinocytes, while the avian corneous cell envelope is probably simpler in comparison to that of mammalian corneocytes (MENON & MENON, 2000; KALININ et al., 2002). Only future biochemical work will allow identification of specific avian proteins involved in sebokeratinocytes differentiation.

The similarity between corneocytes in crocodilian hinge regions and sebokeratinocytes of apteric epidermis allows formulating some speculations about the evolution of apteric epidermis from pro-avian ancestors (Fig. 5 A). Apteric areas perhaps derived from the progressive expansion of the surface of hinge regions and the concomitant reduction of the dorsal surface of scales to bumps (Fig. 5 B). The selection and the progressive spreading of interfollicular/apteric epidermis to broader areas among scales of proto-avian reptiles might have been a major trend toward the origin of the avian integument. The columnar epidermal cells of the basal layer of the bumbs (resembling a placode) might have been connected to a group of mesenchymal cells forming a dermo-epidermal complex (Figs 5 A-B). The molecular basis of this dermo-epidermal association in reptilian skin is unknown (ALIBARDI, 2004b).

The contraction of the basal cells of the epidermis within progressively smaller bumps or cones (located toward the tip of the original scales, see sequence in Figs 5 B-C) might have determined an apparent condensation of dermal cells connected to the epithelium. The interfollicular and the apteric epidermis located around the cones and protofeathers (Figs 5 C-D) maintained the characteristics of hinge regions epidermis, in particular lack of keratohyalin and formation of thin alpha-keratin cells. This may explain the absence of keratohyalin and of a granular layer in the derived apteric epidermis.

The lengthening of the bumps into cones (Figs 5 C-D), together their associated dermis was at the origin of protofeathers (CHUONG et al., 2000; PRUM, 2002). The epidermis of the cones was formed by beta-keratin synthesizing cells organized in a circular region that might have functioned similarly to a collar region (the beta-keratin producing region at the base of a feather follicle in extant birds, see Fig. 5 E). The progressive restriction in the localization of the collar region and associated mesenchyme at the base of proto-feathers and the downward

proliferation of the collar might have produced the follicle (Figs 5 D-E).

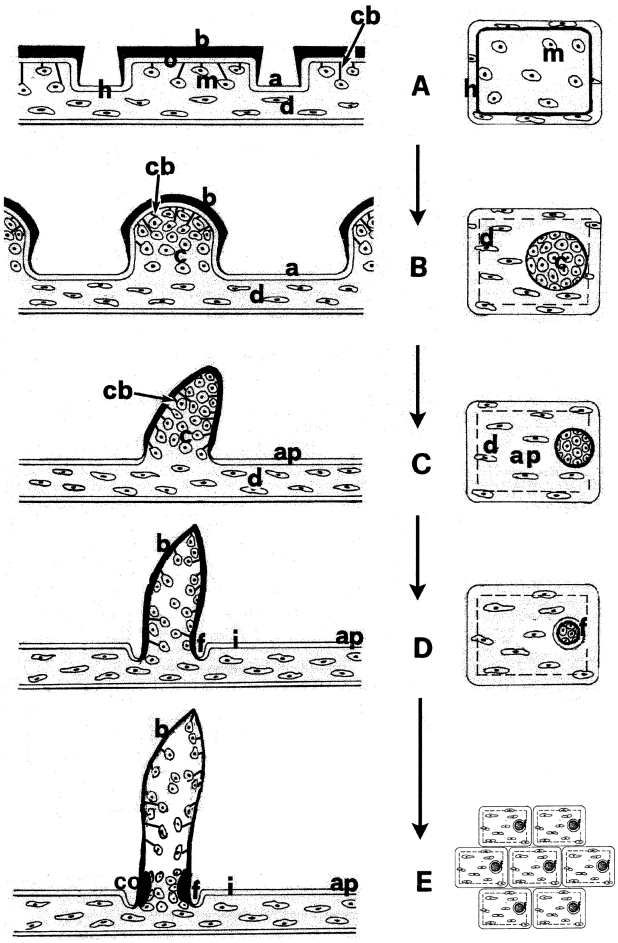

Fig. 5. – Schematic drowing illustrating five hypothetical stages (**A**-**E**) for the origin of the apteric epidermis and protofeathers from an archosaurian scaled epidermis (on the left are the histological sections; on the right are their dorsal view showing the progressive localization of the evolving protofeather toward the original scale tip). **A**, scales of an hypothetical archosaurian progenitor where mesenchymal fibroblasts are joined to the epidermis by cytoplasmic bridges; **B**, tuberculate scale of a protoavian archosaur; **C**, conical scale of a protoavian archosaur; **D**, elongating conical scale with forming follicle. Mesenchymal fibroblasts associated to the collar progressively reduce in the lower part of the cone; **E**, protofeather within a follicle (the dorsal view of 7 protofeathers together the area originally occupied by 7 scales shows the exagonal arranging present in pterylae tracts). Mesenchymal fibroblast are connected to the collar at the base of the protofeather. **Legends** : a, alpha-keratin layer; ap, apteric epidermis (outside interfollicular epidermis of pterylae epidermis); b, beta-keratin layer; c, dermal condensation of mesenchyme; cb, cytoplasmic bridges between mesenchyme and epidermis; d, fusiform fibroblasts of the dense/normal dermis; f, follicle; h, hinge region; i, interfollicular epidermis; m, mesenchymal fibroblasts of the loose dermis; o, outer scale surface. The dashes lines represent epidermal areas of the original scales.

The soft and wrinkly apteric epidermis seems to be associated with the need to maintain a very elastic and flexible tissue among feathers that favors their movement (Homberger & De Silva, 2000). Due to the limited importance of apteric epidermis in the mechanical protection of the body, cornification is reduced in this type of epidermis. The plumage takes up most of the mechanical protection (covering by flattening the feathers over the apteric epidermis), and acts as thermal insulation, leaving to the apteric epidermis a major role in the control of water loss (Lucas & Stettenheim, 1972; Menon et al., 1996; Menon & Menon, 2000).

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Description of the karyotype of *Heimyscus fumosus* and of several other murids from the Mount Doudou area (Gabon)

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ABSTRACT. A first inventory of south-western Gabon forest rodent diversity was realised in the Mount Doudou AERF using cytotaxonomic analysis. The C-banded karyotypes of some *Heimyscus*, *Hylomyscus*, *Praomys*, *Hybomys* and *Malacomys* species are presented. Two karyotypes are here described for the first time : those of *Heimyscus fumosus* and *Hylomyscus sp*. The results of this chromosomal analysis increase the murine specific diversity recognized in central Africa : there probably exists a new species of *Hylomyscus* in the Mount Doudou area.

KEY WORDS : Rodentia, Murinae, karyotypes, C-banding, *Heimyscus fumosus.*

INTRODUCTION

One of the most striking features of tropical Africa is the diversity of its mammalian fauna, especially of the small mammals (DELANY & HAPPOLD, 1979). Since the 60's numerous studies were therefore conducted on small mammals, especially rodents, in central Africa which allowed : 1) to describe new species (e.g. BROSSET et al., 1965; DUBOST, 1965), 2) to identify sibling species, as in the genus *Hylomyscus* (ISKANDAR et al., 1988), and 3) to inventory many sites from Nigeria (e.g. HAPPOLD, 1987), Cameroon (HUTTERER et al., 1992; COLYN et al., 1996), Gabon (e.g. BROSSET et al., 1965; COLYN, et al., 1996), Republic of Congo (e.g. GRANJON, 1991; COLYN et al., 1996), Democratic Republic of Congo (e.g. COLYN & DUDU, 1986; DUDU, 1991; LEIRS et al., 1999), Central African Republic (PETTER & GENEST, 1970; BARRIÈRE & NICOLAS, 2000) and Uganda (e.g. DELANY, 1971; CLAUSNITZER & KITYO, 2001). However, despite this rather large number of studies, African small mammal diversity is not yet fully understood, and the biodiversity of some regions is still little known. In particular this is the case of south-western Gabon, where no precise inventory has been undertaken yet.

The cytotaxonomic analysis constitutes a powerful discriminating tool for screening faunistic diversity of small mammals in general, and rodents in particular (PETTER, 1971; ROBBINS & BAKER, 1978; ROBINSON, 2001); it has allowed taxonomists to reveal the presence of numerous sibling species (e.g., *Arvicanthis* : DUCROZ et al., 1997; *Mastomys* : GRANJON et al., 1997; VOLOBOUEV et al., 2001; *Otomys* : TAYLOR, 2000), which are morphologically similar but are showing sufficient chromosomal divergence to ensure reproductive isolation (see KING, 1993). Indeed, following early works of Matthey dating from the 60's (e.g. MATTHEY, 1959; 1963; 1965; 1967), ∝-systematics in African rodents has experienced a significant renewal greatly due to the input of cytogenetics (PETTER, 1971; ROBBINS & BAKER, 1978; TAYLOR, 2000; ROBINSON, 2001).

MATERIAL AND METHODS

An international project, funded by the WWF (project GA085300), was carried out between March 2000 and March 2001 in the Mount Doudou AERF ("Aire d'Exploitation Rationnelle de Faune"), South-western Gabon, to provide data on the small mammals diversity. The Mount Doudou AERF covers 332 000 hectares, and its altitude ranges from 110 to 700 m A.S.L. A one year study on small mammal community ecology was conducted in its eastern part (02°09S-10°30E), in mostly undisturbed lowland forest (110 m A.S.L.). Ten of the rodents captured during this study were subject to chromosomal analysis.

The chromosomal formula of these specimens (diploid number, 2n and autosomal fundamental number, NFa) was determined on the preparations obtained from fibroblast cell cultures using standard Giemsa staining and C-banding technique (SUMNER, 1972). The fibroblast cell lines are cryopreserved and available, as well as the ethanol preserved tissues at the cell and tissue collections of the Laboratoire Zoologie Mammifères et Oiseaux, Musée National d'Histoire Naturelle, Paris. The collection numbers indicated here refer to this "cell and tissue collection". Skulls were extracted and cleaned for species identification and bodies were preserved in 10% formalin. Both bodies and skulls are stored in the general collections of the Musée National d'Histoire Naturelle, Paris.

RESULTS AND DISCUSSION

Heimyscus fumosus (2001-64 : male (M), and 2001-76 : female (F))

Although originally described as belonging to *Hylomyscus* (Brosset et al., 1965), it was later treated as a species belonging to a distinctive genus (Misonne, 1969). It seems to be present only in the central African lowland forests that are located between the Sanaga and the Oubangui-Congo rivers (Nicolas et al., 2003). The karyotype of *H. fumosus* is described here for the first time (Fig. 1). It is characterized by a diploid number (2n) of 40 and contains 5 pairs of meta- or submetacentric and 14 pairs of acrocentric autosomes thus giving NFa=48. Both sex chromosomes are metacentric, the X chromosome is the largest in the set and the Y chromosome is comparable in size with the largest pair of autosomes. The autosome heterochromatine is situated only in the pericentromeric region, the chromosomes short arms are all euchromatic (Fig. 1). The Y chromosome displays some heterochromatic regions.

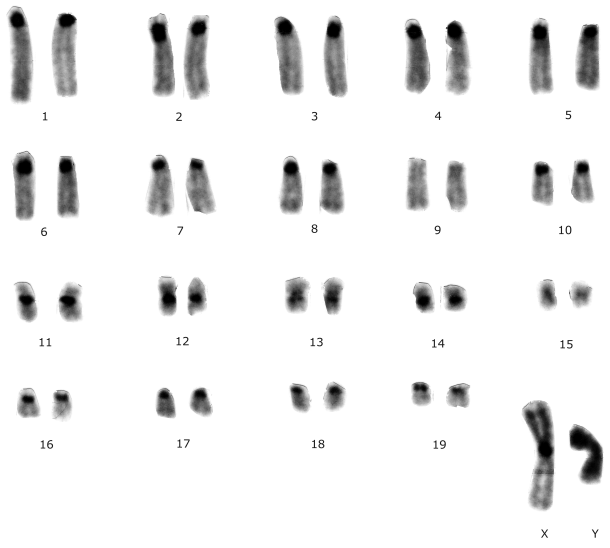

Fig. 1. – C-banded karyotype of *Heimyscus fumosus* (M : n°2001-064).

Hylomyscus

The systematics of this genus is very unclear due to the absence of clear diagnostic external morphological characters (review in Robbins et al., 1980). Since the first chromosomal studies (e.g. Matthey, 1963; 1967) it appeared that karyotype may be a useful discriminating character for at least some species. In the sample studied here two distinct karyotypes were found.

H. cf. stella (2001-98 : F, 2001-99 : F, 2001-100 : F)

These specimens are morphologically similar (skull and external characters) to *H. stella*, the species largely distributed in African rain forest (type locality in East Democratic Republic of Congo, Ituri forest). However the molecular data show that West central African specimens (Cameroon, Central African Republic, Gabon, Republic of Congo) are quite divergent from the East African specimens (Kenya, Democratic Republic of Congo : Lecompte, 2003; Nicolas, 2003). The karyotype of these three specimens has 2n = 46 and NFa = 68 (Fig. 2) and is similar to that earlier described for *H. stella* in Cameroon, Central African Republic and Gabon (Matthey, 1967; Viegas Péquignot et al., 1983; Iskandar et al., 1988; Robbins et al., 1980). The autosome heterochromatine is situated only in the centromeric region, the autosomes short arms are euchromatic whereas the X chromosome short arms are heterochromatic (Fig. 2).

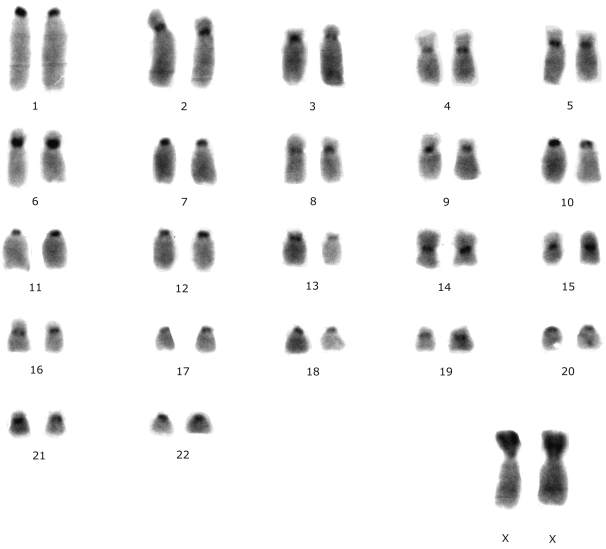

Fig. 2. – C-banded karyotype of *Hylomyscus stella* (F : n°2001-100).

In addition to the karyotype with NFa = 68, Matthey (1963) found NFa = 70 in the same locality (Pointe Noire, Congo). The same value of NFa was found in one specimen from Cameroon (Robbins et al., 1980). Interestingly, morphologically similar animals from Burundi showed a karyotype with 2N = 48 and NFa = 82, which is rather distinct from that described here and in earlier studies (Maddalena et al., 1989). However, considering that the specimens of our study possess only NFa = 68, the nature of this variation remains unknown: The possibility of heterochromatic arms as a source of NFa variation is possible, while no heterochromatic arms have been identified in our sample, representing the lowest autosomal fundamental number known. In order to identify the nature of this karyotypic variation, it is necessary to study specimens from all the distribution area using C- and G-banding pattern. Such study will allow clarifying the taxonomic status of the karyotypic (and molecular) variants of *H. stella*.

Hylomyscus sp (2001-65 : F)

This specimen is morphologically close (skull and external characters) to *H. aeta*, however the interorbital constriction shape as well as palatal foramina are slightly different, and the mammary formula is 2+2 = 8 instead of

1+2 = 6. Its karyotype is characterized by 2n = 56 and NFa = 86 (Fig. 3). The autosome heterochromatine is only situated in the centromeric region (Fig. 3). The X chromosomes show a large band of heterochromatine, including most of the short arms of the X chromosome.

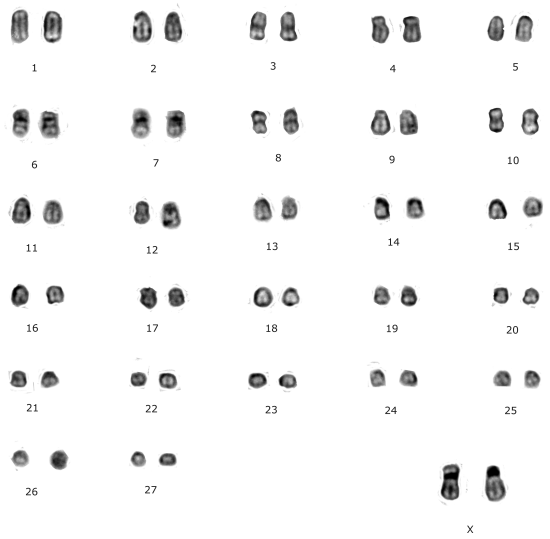

Fig. 3. – C-banded karyotype of *Hylomyscus sp*. (F : n°2001-065).

This karyotype is closer to a series of karyotypes ascribed to *H. aeta* (MATTHEY, 1967; ROBBINS et al., 1980), and differs from the chromosomal formulae classically found in *Hylomyscus* species with 2n ranging between 44 and 48 (MATTHEY, 1963; ROBBINS et al., 1980; VIEGAS-PÉQUIGNOT et al., 1983; ISKANDAR et al., 1988; MADDALENA et al., 1989). The karyotype of *H. aeta* was described for the first time by MATTHEY (1967) for a male specimen from Fernando Pô (Bioko) as having 2n = 52 and NFa = 78, with a metacentric X and an acrocentric Y chromosomes being the largest in the set. However ROBBINS et al. (1980) found two specimens in Cameroon identified as *H. aeta* possessing 2n = 54 and NFa = 86. As a result at least three rather distinct karyotypes have been ascribed to *H. aeta*. Although it is difficult in the absence of chromosome banding data to characterize the exact nature of the chromosomal differences it is clear that this karyotypic diversity most probably hides an unknown taxonomic diversity thus urging for new cytogenetic and molecular studies.

Praomys sp
(2001-63 : M and 2001-77 : M)

These *Praomys* specimens are morphologically identical (skull and external characters) to *P. petteri* (VAN DER STRAETEN et al., 2003). They are characterized by 2n = 42, NFa = 40. Both sex chromosomes are metacentric, the X chromosome is comparable in size with the largest pair of autosomes. This karyotype was already described by MATTHEY (1963) after the analysis of a single specimen from Pointe Noire (Congo). The C-bands allow to identify six autosomes (pairs 1, 2, 3, 6 7 and 8) showing large heterochromatine blocks with a distribution pattern that is highly specific (Fig. 4). These heterochromatine blocks are situated right under the centromeric region and covers between ¼ to ½ of the chromosome length. The heterochromatine pattern is pericentromeric for all the other chromosomes.

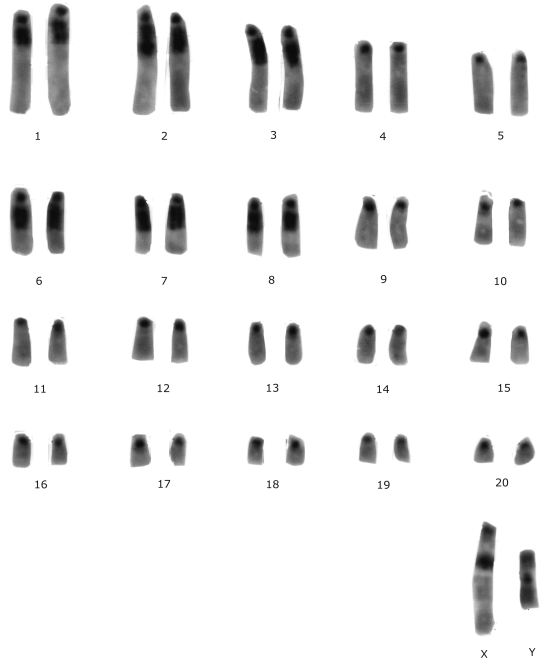

Fig. 4. – C-banded karyotype of *Praomys sp*. (M : n°2001-063).

In the western part of central Africa, three *Praomys* species are identified : *P. jacksoni* (2n = 28; NFa = 26), *P. tullbergi* (2n = 34; NFa = 32) and *P. petteri* (2n = 42; NFa = 62). As our specimens, the karyotype of *P. petteri* is characterized by a diploid number of 42 but contains 11 pairs of meta- or submetacentrics and 9 pairs of acrocentric autosomes thus giving NFa = 62 (MATTHEY, 1965). It would be very interesting to compare specimens of *P. petteri* with those from Gabon, morphologically similar, to investigate the heterochromatine pattern and the presence of such heterochromatine blocks using C-banding. This will allow to verify if the short arms of *P. petteri* metacentric chromosomes are heterochromatic or not and thus to identify the cause of the NFa variation. This could allow a conclusion about the taxonomic status of these new specimens from Gabon. But, as it is for the moment, we know that 1/ the X chromosome is much bigger than the X in *P. petteri*, which is metacentric; 2/ that the Y in our Gabon specimens is metacentric, whereas it is acrocentric in *P. petteri*. These differences seem sufficient to suggest that the specimens from Gabon could be a distinct species. A revision of the genus *Praomys*, combining both molecular and morphometrical data, is presently being investigated in order to solve this problem.

Hybomys univittatus
(2001-72 : F)

The karyotype of the specimen trapped in Mount Doudou is characterized by 2n = 44, NFa = 46. All the chromosomes are acrocentric except for a small pair of metacentric ones. This karyotype is the same as the one described by VERHEYEN & VAN DER STRAETEN (1985) for

H. univittatus from Cameroon. The type locality of *H. univittatus* is in Gabon (Dongila), i.e. in the same faunal region as our study area.

VERHEYEN & VAN DER STRAETEN (1985) analysed three species in the genus *Hybomys* : *H. trivirgatus* (Côte d'Ivoire) 2n = 40 NFa = 38 ; *H. univittatus* (Cameroon) 2n = 44 NFa = 46 ; *H. lunaris* (Rwanda) 2n = 48 NFa = 48. MATTHEY (1959) studied *Hybomys* species labelled *univittattus* in his paper, following VIEGAS-PÉQUIGNOT et al. (1983 ; 1986), characterized by 2n = 48 NFa = 48. This specimen was trapped by Misonne in the Democratic Republic of Congo (F. PETTER, pers. comm.). The karyotype described by MATTHEY (1959) is similar to the *lunaris* karyotype from VERHEYEN & VAN DER STRAETEN (1985) and this specimen from RDC may be a *lunaris* and not a *univittatus* as initially proposed.

Malacomys longipes (2001-70 : F)

The karyotype of this species is characterized by 2n = 48, NFa = 48. All the autosomes are acrocentric except the smallest pair which is metacentric. The X chromosome is a large metacentric one. The karyotype of our study corresponds to that described earlier by VIEGAS-PÉQUIGNOT et al. (1983) from Ivory Coast. The type locality of *M. longipes* is in Gabon (Gaboon river, vicinity of Ogooué), i.e. in the same faunal region as the Mount Doudou area.

CONCLUSION

This study allowed realising a first inventory of forest rodent diversity of the south-western Gabon. Two karyotypes are here described for the first time : those of *Heimyscus fumosus* and *Hylomyscus sp*. The result of this chromosomal analysis increases the murine specific diversity recognised in central Africa : there is probably a new species of *Hylomyscus* in the Mount Doudou area (*Hylomyscus sp* with 2n = 56). Moreover, this study also revealed some systematic problems within *Hylomyscus* and *Praomys* genera.

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Early interaction between deep and superficial layers in avian blastodiscs : uptake of ooplasmic determinants

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ABSTRACT. Parts from radially symmetric prelaid quail blastodiscs placed in culture in contact with isolated cranial quadrants from unincubated chicken blastoderms induce in the chicken upper layer the formation of a miniature embryo with primitive streak and neural plate. Both components (blastodisc parts and upper layer fragments) do not differentiate into an embryo when cultured apart. We concluded that the formation of the avian blastoderm is the result of an interaction between the deep part of the blastodisc and the uncommitted upper layer. Indeed during the culture period the deep part of the quail blastodisc differentiates into quail sickle endoblast and quail junctional endoblast, indicating the formation of Rauber's sickle material in the quail tissue which by induction gives rise to the development of an embryo in the neighbouring upper layer. The here observed induction phenomena result from placing early deep material on more advanced upper layer. This suggests an analogy with the phenomena observed after the eccentric, radially-symmetry-breaking displacement of the deep layer, with reference to the superficial layer which occurs during normal bilateral symmetrization under influence of gravity in undisturbed eggs *in utero*.

KEY WORDS : avian blastoderm, gastrulation, neurulation, Rauber's sickle, symmetrization, δ and γ ooplasms, ooplasmic determinants.

INTRODUCTION

From the experimental studies of LUTZ (1953), VINTEMBERGER & CLAVERT (1954) CLAVERT (1960), KOCHAV & EYAL-GILADI (1971) we know that the avian blastodisc before laying presents a radial symmetry. Studies (CALLEBAUT 1993a, b, c; CALLEBAUT & VAN NUETEN, 1994, 1995; CALLEBAUT et al., 1998) yielded new data about the structure and developmental events in avian intrauterine germ discs during bilateral symmetrization and in unincubated eggs. In the present article we also use the name blastodisc for an intrauterine germ disc. We have shown by the use of the quail chicken chimera technique that Rauber's sickle (RAUBER, 1876) is the early gastrulation organizer in avian blastoderms (CALLEBAUT et al., 1997a). During early incubation it only differentiates into : 1) junctional endoblast by local proliferation of its cells into the neighbouring subgerminal ooplasm; (CALLEBAUT et al., 2000c) and 2) sickle endoblast by centripetal and cranial growth, forming a one-cell-thick layer (CALLEBAUT & VAN NUETEN, 1994; CALLEBAUT et al., 1997b). Although Rauber's sickle and the Rauber's sickle-derived sickle endoblast and junctional endoblast have a very important and indispensable inductive function for the development of the embryonic tissues during gastrulation and neurulation (CALLEBAUT et al., 2003), they never give rise to cells of the embryo proper and therefore belong to the so-called extraembryonic part of the blastoderm (as is also the case for the Nieuwkoop center in amphibians : GUGER & GUMBINER, 1995). In conclusion, the avian Rauber's sickle fulfils the major postulate to be homologous with a functional Nieuwkoop center (NIEUWKOOP, 1969, 1973), namely, the potential for organizer induction without itself contributing to the new structure. Recently, this has been confirmed by molecular biology studies of goosecoid genes in avian blastoderms. Indeed, LEMAIRE et al. (1997) found strong expression of one of the goosecoid genes (GSX) in the upper layer above Rauber's sickle, suggesting induction by the latter. Thus, goosecoid expression was not found in Rauber's sickle, but above it. Later, these upper layers will ingress and form the primitive streak and Hensen's node, which are also GSX-expressing. The latter are close to the amphibian organizer in SPEMANN & MANGOLD'S definition (1924). Thus, Rauber's sickle and not Hensen's node (which develops much later as a complex secondary structure) is the early organizer of the avian embryo (CALLEBAUT & VAN NUETEN, 1994; CALLEBAUT et al., 1996, 1998). Moreover, during the peripheral migration of mesoblast, Rauber's sickle induces the formation of blood islands (CALLEBAUT et al., 2000a) and associated coelomic vesicles, followed by the development of vitelline blood vessels with primitive blood cells and the coelomic cavity including the heart and pericard (CALLEBAUT et al., 2004a). After culture of isolated chicken cranial quadrants (from unincubated avian blastoderms) always a preneural plate forms (CALLEBAUT et al., 2000b). Sections of the latter show a pronounced thickening of the upper layer above the deep layer composed of segregated endophyll. On sections the thickened UL is separated from the endophyll by a large space, and presents localized "banding" of the nuclei (ENGLAND, 1973) indicating primary neural induction (ENGLAND & LAWSON, 1993) in the absence of chordamesoderm (CALLEBAUT & VAN NUETEN, 1995). The latter developmental pattern with only the formation of a preneural plate was also seen after the culture of cranial quadrants from unincubated quail blastoderms (CALLEBAUT & VAN NUETEN, 1995). In a part of the isolated cra-

nial quadrants also a more or less developed primitive streak was seen. The reason for this different developmental behaviour was the occasional presence (proximity) or absence of Rauber's sickle horns in the cranial quadrants. Whole egg yolk balls from quail eggs extracted before bilateral symmetrization (in a white calcareous shell) develop normally during incubation in a humid atmosphere (CALLEBAUT, 1993a). Even when cultured *in toto* in egg white outside their egg shell, they will still develop (CALLEBAUT 1991; CALLEBAUT et al., 2000b). By contrast in the present study, we observed that blastodiscs (or part of them) extracted before bilateral symmetrization and separated from their subgerminal ooplasm and their egg yolk ball, do not develop when cultured *in vitro*. However parts of these unsymmetrized quail blastodiscs, placed in contact with the upper layer of isolated cranial quadrants from unincubated chicken blastoderms, induce a miniature embryo. In the present study we try to explain these phenomena by comparing them with the normal early interaction *in ovo* between upper and deeper part of the avian blastodisc.

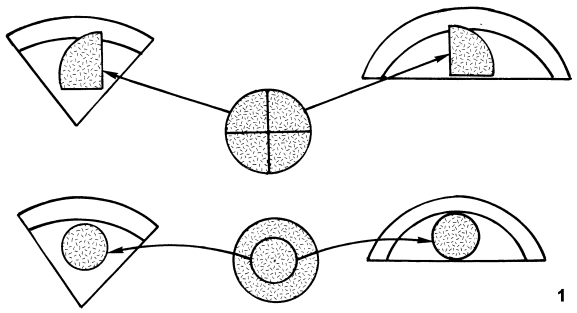


Fig. 1. – Schematic representation of the transplantation of fragments (quadrants or only the central part) of radially symmetric extracted quail blastodiscs (dotted seen in the middle region of the figure) on cranial chicken quadrants (left of the figure) or an anti-sickle regions (right of the figure).

MATERIAL AND METHODS

The used quail (*Coturnix coturnix japonica*) eggs were extracted from the uterus at the beginning of calcification i.e. approximately 10h after oviposition of the previous egg, according to STEPINSKA & OLSZANSKA (1983). From these extracted quail eggs the blastodiscs were removed and the whole blastodisc or parts of it were incubated *in vitro* according to the culture method of NEW (1955) or SPRATT (1947). Other quail blastodiscs were sectioned in quadrants or only, the central part of it was isolated (Fig. 1). These quadrants or central parts were then placed in culture with their deep side on the deep side of the isolated cranial quadrant or anti-sickle region of an unincubated chicken blastoderm. In a similar way they were cultured *in vitro*. Stereomicroscopic polaroid photographs were taken always in the same direction, at the beginning, during and at the end of the culture period (23-28h). Fixation was performed overnight in a modified Heidenhain's fixative (ROMEIS, 1948) containing 0,5g sodium chloride, 2g trichloric acid, 4ml acetic acid, 20ml formalin and 80ml water. After rinsing in tap water the blastoderm associations were placed *in toto* in Unna solution. After rapid dehydration in a graded alcohol series and embedding in paraffin wax they were sectioned perpendicular to the visible or presumed axis. The UNNA staining permitted eventual observation of the orientation of the embryo in the paraffin wax before sectioning. The deparaffinized 8 µm thick sections were Feulgen-stained after DEMALSY & CALLEBAUT (1967) in order to be able to identify the origin of the nuclei in the chimeric blastoderms. This allowed us to observe the typical central or subcentral chromatin granule in the nuclei of the grafted quail cells (CALLEBAUT, 1968; KOSHIDA & KOSIN, 1968; LEDOUARIN & BARQ, 1969) as well as to observe their relationship with the chicken tissues.

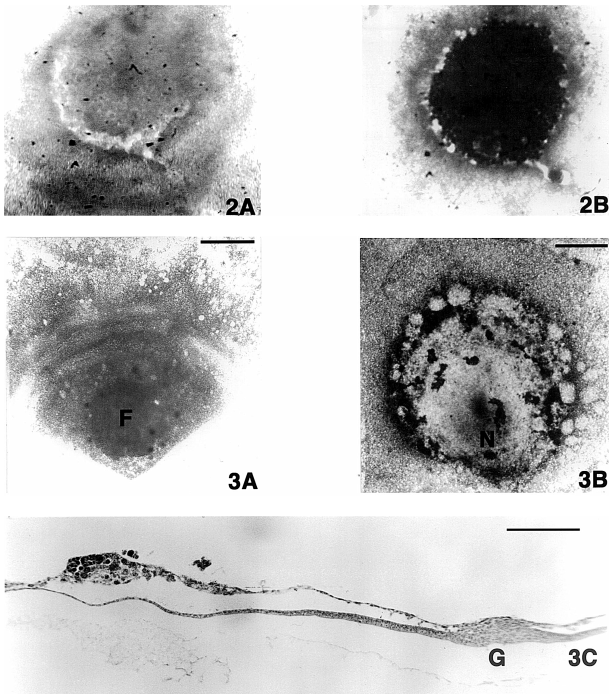


Fig. 2A. – Micrograph of a whole extracted prelaid radially symmetric quail blastodisc at the start of the culture (same magnification as Fig. 3A).
Fig. 2B. – The same blastodisc as seen in Fig. 2A. after 1 day of culture : no developmental phenomena have taken place; the disc has a denser aspect now (same magnification as Fig. 3A).
Fig. 3A. – On the cranial quadrant of an unincubated chicken blastoderm a central circular fragment (F) of a prelaid radially symmetric quail blastodisc has been placed; bar : 1mm.
Fig. 3B. – The chimera of Fig. 3A. after approximately 1 day of culture : note the development of a radially directed miniature embryo with visible neural plate (N) and primitive streak in its prolongation; bar : 1mm.
Fig. 3C. – Feulgen stained section through the embryo seen in Fig. 3B. shows the presence of a fully developed primitive streak with groove (G); bar : 200µm.

RESULTS

Culture of whole radially symmetric intrauterine quail germ discs (blastodiscs after extraction from the uterus) (n = 6)

Such a blastodisc at the start of the culture period is seen in Fig. 2A. After 23h of culture, no differentiation or

growth is observed (Fig. 2B). On sections nor a neural plate, nor a primitive streak is seen indicating the total absence of induction phenomena.

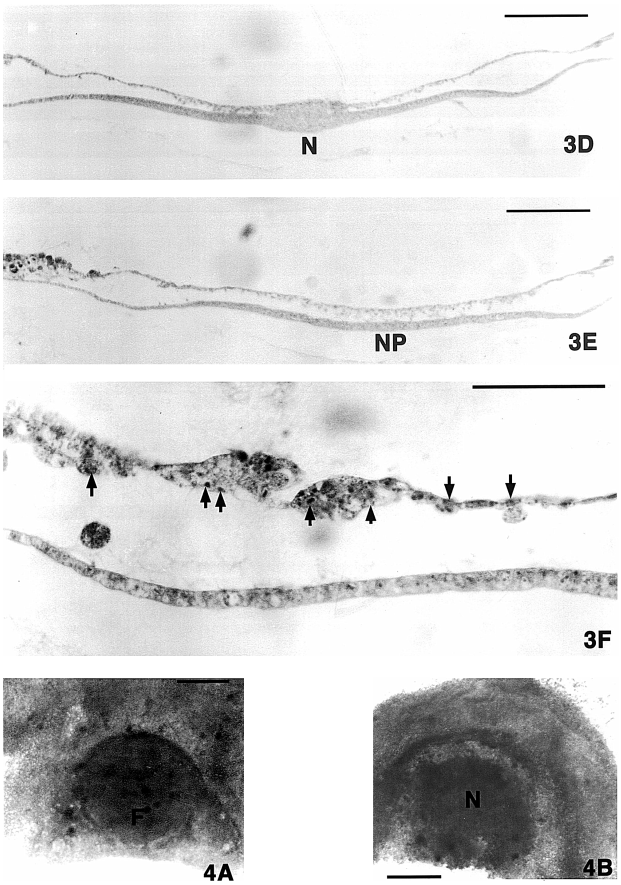


Fig. 3D. – Section through the nodus region (N) of a similar embryo as in fig. 3B.; bar : 200µm.
Fig. 3E. – Section through the neural plate (NP) region formed in the embryo of Fig. 3B.; bar : 200µm.
Fig. 3F. – Feulgen stained section through the primitive streak forming area of the embryo of Fig. 3B. Note laterally the voluminous junctional endoblast containing quail cells (indicated by upside directed arrows) and the flat one cell thick more medial sickle endoblast also containing quail cells (indicated by downside directed arrows); the upper chicken layer (epiblast) in which the primitive streak is induced is seen at the bottom of the figure; bar : 100µm.
Fig. 4A. – The central circular part (F) of a prelaid radially symmetric quail germ disc was placed on an isolated anti-sickle region of an unincubated chicken blastoderm at the start of the culture period; bar : 1mm.
Fig. 4B. – The quail-chicken chimera of Fig. 4A. after 20h of culture : a dense (pre)neural plate (N) has formed (confirmed by sectioning); bar : 1mm.

Placing the deep side of a fragment of a similar unsymmetrized quail blastodisc on the deep side of a chicken cranial quadrant in culture (Fig. 3A) (n = 14)

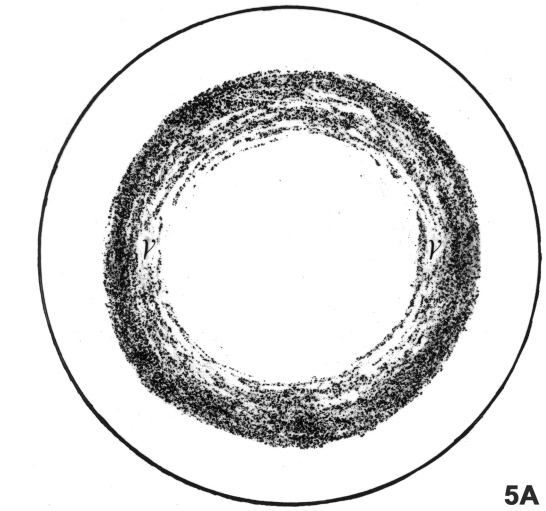
After culture a miniature embryo with a radially placed axis is observed (Fig. 3B). On sections this axis is seen to be partially formed by a primitive streak (Fig. 3C) with a more or less developed nodus (Fig. 3D). In other serial sections a neural plate localized centrally or peripherally (or both) form the primitive streak area, can be discerned (Fig. 3E). The primitive streaks are formed from chicken tissue and are seen to be in association with quail tissue. They are formed in the median part of the upper layer of the area limited by V-shaped quail junctional endoblast (derived from the locally developed Rauber's sickle (Fig. 3F). Quail sickle endoblast (Fig. 3F) is seen laterally from the primitive streak, as the result of the displacement by the concentrically expanding definitive chicken endoderm derived from the tip of the primitive streak. Central fragments of quail germ discs give in general a better embryonic development than quadrants.

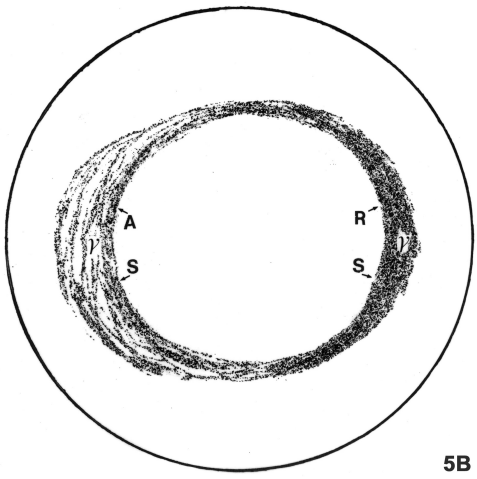
Placing a fragment of radially symmetric quail blastodisc on the deep side of a chicken anti-sickle region in culture (n = 8) (Fig. 4A)

In half of this associations a preneural plate was induced (Fig. 4B) but no primitive streak developed. In another half of the cases, no induction phenomena were observed. Here also a central part excised from a quail germ disc, induces more prominent phenomena in the chicken upper layer.

DISCUSSION

Our results show that whole radially symmetric extracted quail germ discs do not develop *in vitro* in conditions which permit normal development of bilaterally symmetrized unincubated blastoderms. By contrast a fragment of a similar radially symmetric quail blastodisc, placed with its deep side on the deep side of a chicken cranial quadrant, or anti-sickle region differentiates and induces gastrulation and neurulation phenomena. The miniature embryo which so develops is formed by a concerted action between both fragments of different origin and developmental stage. A circular centrally excised fragment of quail germ disc gives the best final development. That these central regions have a more powerful inducing effect on the chicken upper layer is probably the consequence of the presence of a greater part of nucleus of Pander material (containing δ-ooplasm surrounded by some γ-ooplasm : CALLEBAUT, 1987). The localization of the δ and surrounding γ ooplasm in the radially symmetric quail blastodisc and neighboring underlying ooplasm is represented schematically in Fig. 5A. After bilateral symmetrization, in the unincubated blastoderm, Rauber's sickle material is found in the γ ooplasm of the future caudolateral part of the germ disc (Fig. 5B). These ooplasmic layers extend vertically to the upper layer. In the diametrically oposed anti-sickle region the γ containing ooplasm is disrupted from the future cranial part of the blastoderm (which contains no γ ooplasm) (Fig. 5C). The ooplasmic layers in the future cranial region are horizontally disposed and are less condensed then in the caudal Rauber's sickle region as the result of the eccentric tilting of the ooplasm and egg yolk (CALLEBAUT, 1993a, b, c). In a recent study we have seen that the nucleus of Pander (before bilateral symmetrization) has a preneurulation and sometimes a concommited gastrulation effect (CALLEBAUT et al., 2004b). We know that the first cleavage furrows penetrate deeply in the superficial part of the





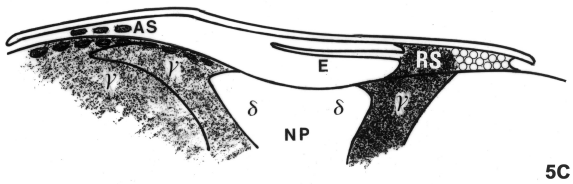


Fig. 5A. – Schematic representation of the circular localization (seen in vertical projection) of the γ ooplasm (γ) in the avian blastodisc before bilateral symmetrization. The γ ooplasm surrounds the more central δ ooplasm (white in the figure).
Fig. 5B. – Schematic representation of the ovoid localization of the γ ooplasm (seen in vertical projection) in the bilateral symmetrized blastoderm; the condensated Rauber's sickle (RS) and the expanded anti-sickle region (AS) have eccentrically formed in the γ ooplasm which surrounds the δ ooplasm.
Fig. 5C. – Schematic representation (simplified after Callebaut, 1993b) of the localization of the γ and δ ooplasm in a mediosagittal section of an unincubated avian blastoderm (bilaterally symmetrized) : caudally in the neighborhood of the Rauber's sickle (RS) the layers in the γ ooplasm are condensed vertically and adhere to the upper layer, whilst cranially in the anti-sickle region (AS) the layers are horizontally flattened and are not taken up by the blastoderm; E : endophyll and NP : nucleus of Pander both containing δ ooplasm (δ).

nucleus of Pander (composed of δ ooplasm) which gives rise to also δ ooplasm-containing endophyll and primordial germ cells (Callebaut, 1984). Preneurulation phenomena appear initially in the neighbourhood of δ ooplasm-containing structures i.e. nucleus of Pander and endophyll. The δ ooplasm seems thus to play an early role in (pre)neurulation phenomena, while the more peripheral and more superficial γ ooplasm influences early gastrulation. In the sickle-shaped region where the γ ooplasm, forming Rauber's sickle is taken up by the germ during and after bilateral symmetrization, gastrulation phenomena start with formation of a primitive streak close to the place where the spatially oblique uptake is maximal i.e. in the middle of Rauber's sickle (Callebaut, 1993c, 1994). A similar phenomenon takes place by the oblique formation of the subgerminal space (Callebaut, 1987, 1993b), through the nucleus of Pander (containing δ ooplasm). This makes that endophyll (also containing δ ooplasm) is only taken up in the caudal half of the germ. The latter is responsible for preneurulation phenomena. This indicates the fundamental influence of the uptake of ooplasmic determinants respectively for gastrulation (by γ ooplasm containing cells) and for preneurulation (by δ ooplasm) phenomena. From a comparative literature study, Eyal-Giladi (1997) concludes that the establishment of the axis in chordates (axialization with bilateral symmetrization) depends on the translocation of oocytal (maternal) determinants form the vegetal pole towards the future dorsocaudal side of the embryo. On arrival at their destination the activated determinants form in all chordates, an induction center homologous to the amphibian "Nieuwkoop center" (or avian Rauber's sickle) which later will respectively induce the formation of the "Spemann's organizer" or the avian Hensen's node. Our studies confirm this hypothesis. The placing of a fragment of quail germ disc on a cranial chicken blastoderm quadrant produces a more developed miniature embryo then after the placing on an isolated anti-sickle region. This can probably be explained by the quantity of involved chicken blastoderm. The presence of quail sickle endoblast and quail junctional endoblast in the present experiments demonstrates that both have a preponderant influence in the formation and orientation of the chicken primitive streak. The difference in developmental potencies *in vitro* between an unsymmetrized blastodisc which is removed from its underlying ooplasm and yolk and an unsymmetrized blastodisc which remains on its egg yolk ball seems due to the eccentric dislocation of the upper layer with reference to the underlying ooplasm during the oblique positioning of the egg *in toto* by which γ ooplasm is taken up forming Rauber's sickle (Figs 5B-C). Indeed a similar phenomenon occurs in our present experiments when early radially symmetric quail deep layer material and ooplasm comes in contact with the deep side of the chicken upper layer. So an artificial association of elements from originally differently localized regions takes place, resulting in the differentiation of deep material into Rauber's sickle derived junctional and sickle endoblast which finally organize and dominate the whole induced embryo (Callebaut et al., 2003), without forming part of the final embryonic tissues.

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Habitat differences in the food composition of the wasp-like spider *Argiope bruennichi* (Scop.) (Aranei : Araneidae) in Poland

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ABSTRACT. During the last few decades the wasp-like spider *Argiope bruennichi* Scopoli, 1772 expanded its wide geographical distribution in Europe. In this paper we describe and test differences in the diet composition of the wasp-like spider inhabiting a river valley (traditional habitat) and xerothermic grassland (new habitat) in Poland. From a total of 163 webs of *A. bruennichi*, 430 prey items were found, mainly insects : Coleoptera, Diptera, Homoptera, Heteroptera, Hymenoptera, Lepidoptera, Mecoptera, Odonata, Orthoptera and Neuroptera. Moreover, a semi digested specimen of the common frog *Rana temporaria* was recorded. Habitats differed significantly in the percentage distribution of eleven general food categories. Among potential influencing factors the number of caught prey was correlated only with the height of the web hub above the ground. The wasp-like spider is ecologically flexible in the use of novel food spectra and this probably allows the colonisation of new localities, as well as habitats.

KEY WORDS : *Argiope bruennichi*, diet, prey selection, web structure, frogs.

INTRODUCTION

Amongst orb-weaving spiders the wasp-like spider *Argiope bruennichi* Scopoli, 1772 expanded its geographical range to northern parts of Europe. The range of the wasp-like spider was previously limited to south-east Europe (Poetzsch, 1963 Barabasz & Górz, 1998). However, during the last few decades the species has colonized many new sites in Belgium, France, The Netherlands, Poland, Denmark, Sweden and even Great Britain (Guttmann, 1979; Jonsson & Wilander, 1999; Moyes, 1997; Scharff & Langemark, 1997). The most important factors causing rapid geographical expansion of the wasp-like spider are : climate change, especially the increase in numbers of sunny and dry days in summer, floods of large rivers in Europe, as well as the establishment of large open habitats due to deforestation and drainage (Dziabaszewski, 1959; Guttmann, 1979; Helsdingen, 1982; Puts, 1989; Weickmann & Grobmeier, 1997; Scharff & Langemark, 1997; Linden, 2000).

Similar to other orb-weaving spiders, *A. bruennichi* is a generalist predator (Riechert & Łuczak, 1982; Riechert & Harp, 1987; Malt et al., 1990; Fasola, 1999). However, many studies showed that orthopterous insects and dragonflies were the main prey of the spider (Urbański, 1948; Bednarz, 1966; Nyffeler & Benz, 1978; Barabasz & Górz, 1998). Moreover, Conrad & Breinl (1992) even indicated a strong dependence between rich orthopteran fauna and distribution of *A. bruennichi* and linked the geographical range of the wasp-like spider with orthopteran abundance, as potential food supply. On the other hand, some authors showed that other prey, such as dipterans, hemipterans (mainly aphids) and hymenopterans constituted an important part of the wasp-like spider diet (Nyffeler & Benz, 1982; Malt et al., 1990). However, in publications on wasp-like spider diet only a simple description of food content is given, without linking it to habitat, potential food sources, or web structure.

Therefore, in this study we focused on (1) a description of the wasp-like spider's diet composition in two different habitats in Poland; (2) analyses of differences in prey caught in both habitats; (3) some factors affecting hunting success connected especially with web structure (e.g. stabilimenta). We discuss our findings in the light of colonisation of new areas (*sensu* localities, as well as habitats) in Poland (particularly) and in Europe (generally).

MATERIAL AND METHODS

Studies were carried out during August 1999 in the Wielkopolska province, western Poland (52º N, 16º E). The study area covers two different habitats : wetland in the Warta river valley, near the village of Wrąbczykowskie Holendry (Pyzdry region) and small marginal grassland habitats (arable fields), near the villages : Powodowo, Obra, Daszewice (Wolsztyn and Poznań regions). The density of wasp-like spiders in both habitats is similar, ca 0.3 orb-webs/1m^2.

The spider webs were monitored before noon, mainly 9-11 a.m. The geographical exposition was measured based on the axis of the web elevation to the ground level. To ensure that chosen prey items of *A. bruennichi* were sampled, only wrapped preys were removed from webs and stored in tubes with ethyl alcohol (70%). To explain silk details (stabilimenta and turns on stabilimenta) on webs, Fig. 1 was supplied. Because we did not control all parameters for all webs, sample size is different in various analyses. Standard statistical methods were used to describe and analyse the data (Zar, 1999). All statistical tests were two-tailed. We considered $P < 0.05$ as the minimum acceptable level of significance.

RESULTS

Prey structure

Sampling a total of 163 webs of *A. bruennichi* revealed 430 prey items (mean ± SD = 2.6 ± 1.3 prey/web). In the webs, insects from the following ten orders were represented : Coleoptera, Diptera, Homoptera, Heteroptera, Hymenoptera, Lepidoptera, Mecoptera, Odonata, Orthoptera and Neuroptera. The most numerous species caught in the webs were : Blue-tailed Damselfly *Ischnura elegans* (Van der Linden, 1832) and a squash bug *Coreus marginatus* (Linnaeus, 1758). Moreover, in one web an eleventh category, one semidigested specimen of the common frog *Rana temporaria* (Linnaeus, 1758), was found.

Dragonflies and dipterans contributed over 62% of the total prey captures - Table I.

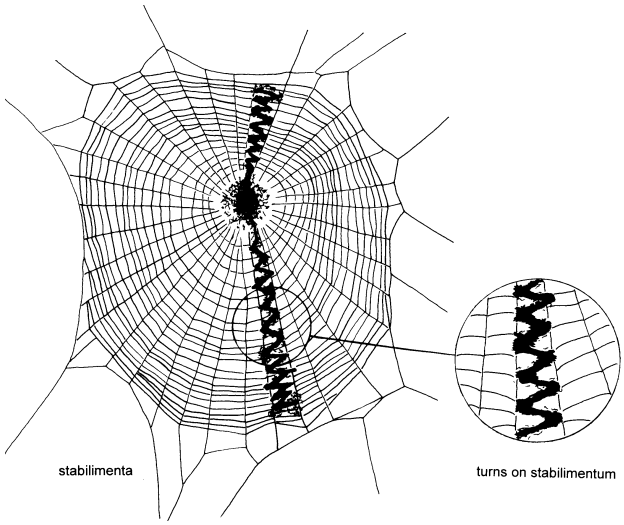

Fig. 1. – Web structure of *A. bruennichi*.

TABLE I

Composition of prey capture species in two different habitats.
Explanations: RV – river valley, XH – xerothermic habitats

Order	Family	Prey items	RV	XH
Coleoptera	Chrysomelidae	*Agelastica alni* (Linnaeus, 1758)	1	
		Cassida vittata Villers, 1789	3	
		Cassida viridis Linnaeus, 1758	1	
		Gastrophysa polygoni (Linnaeus, 1758)		4
		Phyllotreta sp.		2
	Coccinellidae	*Coccinella septempunctata* Linnaeus, 1758	2	
		Tytthaspis sedecimpunctata (Linnaeus, 1761)		2
		Propylea quatuordecimpunctata (Linnaeus, 1758)		1
	Geotrupidae	*Anoplotrupes stercorosus* (Hartmann, 1791)	1	
	Cetoniidae	*Protaetia cuprea* (Fabricius, 1775)	1	
	Staphylinidae		1	
	Phalacridae			1
	Latridiidae			8
Diptera	Bibionidae		1	
	Chironomidae		2	
	Culicidae		3	
	Syrphidae		1	
	other		10	104
Homoptera	Cercopidae		3	30
Heteroptera	Coreidae	*Coreus marginatus* (Linnaeus, 1758)	20	
	Miridae		3	
	Nabidae		1	
	Pentatomidae		6	
	Scutellaridae	*Eurygaster* sp.	3	
	other			26
Hymenoptera	Apoidea		7	
	Argidae		1	
	Formicidae		1	
	other		2	8
Lepidoptera	Pyralidae	*Pleuroptya ruralis* (Scopoli, 1763)		6
	other			11
Mecoptera	Panorpidae	*Panorpa communis* Linnaeus, 1758	4	
Odonata	Coenagrionidae	*Ischnura elegans* (Van der Linden, 1823)	134	
	Libellulidae	*Sympetrum sanguineum* Müller, 1764	5	
	other			6
Orthoptera	Tetrigidae	*Tetrix subulata* (Linnaeus, 1758)	1	
	Tettigoniidae	*Metrioptera roeselii* (Hagenbach, 1822)	1	
Neuroptera	Chrysopidae			1
Anura	Ranidae	*Rana temporaria* (Linnaeus, 1758)	1	

Web site / web attachment substrate

Webs of the wasp-like spider were located on 20 plants. The spider spun their webs most commonly on grasses (Poaceae) and nettle *Urtica dioica* (Urticaceae). We found significant differences in plant species composition used by spiders for web building in the two habitats (Table II, chi-square = 63.0, df = 9, P < 0.0001, calculated only for plant taxa with over 5 spider webs). In the river valley Poaceae, *Gallium* and *Urtica* were used more commonly, whereas in xerothermic grassland : Poaceae, *Oenanthe* and *Carex* were used (Table II).

TABLE II

Plants used as a basis for web in two habitats in western Poland. Explanations – see TABLE I.

Plant	RV		XH		total	
	N	%	N	%	N	%
Achillea	3	2.34			3	1.76
Alnus	4	3.13			4	2.35
Carex	2	1.56	6	14.29	8	4.71
Cirsium	8	6.25			8	4.71
Equisetum	3	2.34			3	1.76
Frangula	5	3.91			5	2.94
Galium	14	10.94			14	8.24
Poacea	27	21.09	21	50.00	48	28.24
Juncus	6	4.69			6	3.53
Lamium	1	0.78			1	0.59
Mentha	8	6.25	4	9.52	12	7.06
Oenanthe		0.00	9	21.43	9	5.29
Plantago	1	0.78			1	0.59
Potentilla	6	4.69			6	3.53
Rhamnus	2	1.56			2	1.18
Rubus	4	3.13			4	2.35
Rumex	1	0.78	2	4.76	3	1.76
Umbelliferae	2	1.56			2	1.18
Urtica	24	18.75			24	14.12
Vicia	7	5.47			7	4.12
No. of plant	19		5		20	

Differences in diet composition between habitats

We found significant differences in diet composition between the habitats (Table I; Fig. 1). In the river valley a higher percentage of dragonflies (63.2%), and lower percentage of dipterans (7.7%) and homopterans (1.4%) and a lack of lepidopterans in the wasp-like spider diet were found. However, in xerothermic grassland the food spectrum consisted of dipterans (49.5%) and hemipterans (26.7%). Habitats differed significantly in the percentage distribution of the eleven general food categories (chi-square = 233.1, df = 10, P < 0.0001).

Factors affecting prey capture

We found no significant relation between the number of prey in the webs and direction of the web geographical exposition (Kruskal-Wallis ANOVA, $H_{8, 40} = 9.508$, P = 0.301). The webs (hubs) were on average (± SD) 54.4 ± 16.0 cm above ground level (range 15-85 cm, n = 40). The number of caught prey was correlated with height of the web hub above ground level (Fig. 2, Spearman rank correlation, $r_s = 0.315$, n = 40, P = 0.048).

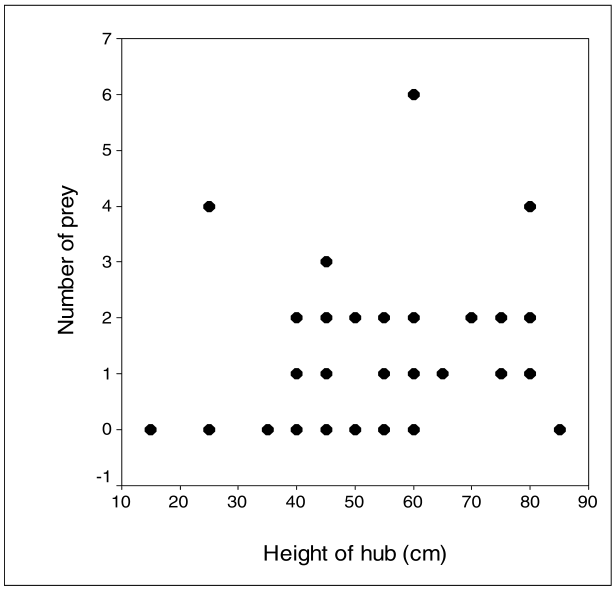

Fig. 2. – Relationship between height of web hub above ground and number of prey caught by the wasp-like spider. Data for both habitats were pooled.

Relationships between the number of prey and web structure

Altogether 40 webs were taken for analysis from both habitats. There were 12.5% of webs with no stabilimentum, 62.5% of webs with one stabilimentum and 25% of webs with two stabilimenta (mean ± SD : 1.13 ± 0.61). Concerning turns on stabilimentum we found the highest frequency of six turns (17.5%) among analysed webs with such decorations (mean ± SD : 7.2 ± 4.6). There was no significant correlation between the number of captured prey and the number of stabilimenta on the web (Spearman rank correlation $r_s = - 0.091$, n = 40, P = 0.578), nor between the number of captured prey and the number of turns on stabilimentum (Spearman rank correlation $r_s = - 0.017$, n = 40, P = 0.917). The webs with different number of stabilimenta did not differ significantly in height above ground level (ANOVA, $F_{2,37} = 2.237$, P = 0.112).

DISCUSSION

The food of web building spiders, including *A. bruennichi*, is mainly selectively filtered from the air (Nentwig, 1987). However, the wasp-like spider traps in the web both small sized insects (dipterans or aphids) as well as larger ones living on plants and/or the ground (grasshoppers or beetles). However, large sized insects even with a lower percentage among prey items can play an important role in providing food biomass for the orb-weavers (Howell & Ellender, 1984). The composition of the diet of orb-weavers included mainly the groups such as Homoptera and Diptera (Nentwig, 1987). *Argiope bruennichi* was known, especially in earlier publications, as a species that specialised in catching large sized invertebrates, mainly grasshoppers (Urbański, 1948; Crome & Crome, 1961; Poetzsch, 1963; Bednarz,

1966; PFLETSCHINGER, 1976). Our findings (a small (0.5%) proportion of orthopteran prey items) suggest that especially in newly occupied habitats, the wasp-like spider could explore a new diet spectrum of Diptera and Hemipterans. NYFFELER & BENZ (1978), obtained similar results during a study of prey selection of the wasp-like spider on fallow land near Zurich (Switzerland). This suggests that the trapping of small prey is a possible adaptation to new habitats where much larger food items, e.g. grasshoppers and dragonflies are strongly limited. Although the wasp-like spider is behaviourally adapted (PASQUET & LEBORGNE, 1990, 1998) to catch orthopterans, the choice of smaller prey is an advantage to expand geographical distribution to the North (INDYKIEWICZ et al., 1995; BARABASZ & GÓRZ, 1998), as well as to occupy new habitats : small patches of xerothermic grassland in intensively used farmland, or even arable field (BARABASZ & GÓRZ, 1998; CICHOCKI, 1998; and our unpublished data).

Moreover, an additional adaptation to the occupation of a new habitat is a change of plant species used as substrate for silk attachment. For a congeneric species, *Argiope aurantia* Lucas, 1833, MCREYNOLDS (2000) found that webs on grasses were more effective in trapping orthopteran prey than those established on herbs and composites. However, we did not test this relationship directly; we can only suggest that the use of a wide range of plants in new habitats improved possibilities of catching novel prey sources.

An important factor improving spider foraging strategy is web structure, especially conspicuous silk structures called stabilimenta (HERBERSTEIN et al., 2000). It is suggested that increased number of stabilimenta can attract prey to the web (STARKS, 2002). HERBERSTEIN (2000), in a study of *Argiope keyserlingi* Karsch 1878, showed that webs with more stabilimenta caught more prey than webs carrying fewer decorations. However, this author did not find a relation between web height above ground and the rate of prey capture. Our findings are in contrast to this. The number of prey caught by the wasp-like spider was correlated with web (hub) height above ground level. We found that higher webs were more successful. Furthermore, we did not find an effect of the number of stabilimenta on the number of prey items in the web. BLACKLEDGE & WENZEL (1999), studying dipterans as prey, obtained interesting results and found that this group of airborne insects were less frequently caught in webs with stabilimenta than in those without stabilimenta. Wasp-like spiders feed mainly on tiny invertebrates on the northern part of their distribution and our results show that Odonata and Orthoptera were caught very seldom. Therefore, this is probably the reason why stabilimenta are not needed to catch most of such spider prey. Selection pressure should cause a significant decrease in the percentage of webs with those ornaments and it should be tested during a long-term study in the future.

We found also the capture and partly ingestion of a small specimen of the common frog by the wasp-like spider. It is, according to our knowledge, the first record of foraging on a vertebrate by *A. bruennichi* (RIECHERT & ŁUCZAK, 1982; RIECHERT & HARP, 1987 and other cited literature). To fully understand the role of food in the colonisation of new localities and habitats this problem should be studied in detail, both by description of diet contents and by field experiments.

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Mitochondrial DNA sequence data suggests two independent colonizations of the Comoros archipelago by Chameleons of the genus *Furcifer*

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ABSTRACT. We used ND4 mtDNA sequences (815bp) to examine the relationships between *Furcifer* chameleons (Chamaeleonidae ; Reptilia) from the Comoro Islands. High genetic divergence between *F. cephalolepis* from Grand Comoro and *F. polleni* from Mayotte is hardly compatible with the hypothesis of them being sister-taxa given the young geological age of both islands. Thus, each island was independently colonized, presumably from Madagascar. Genetic diversity within both islands is similar, despite their very different geological ages. The degree of divergence found within a recent island like Grand Comoro may indicate that the molecular clock calibration typically applied to reptiles is not appropriate for this species.

KEY WORDS : Comoros, *Furcifer polleni*, *Furcifer cephalolepis*, Chamaeleonidae, colonization, ND4, molecular clock.

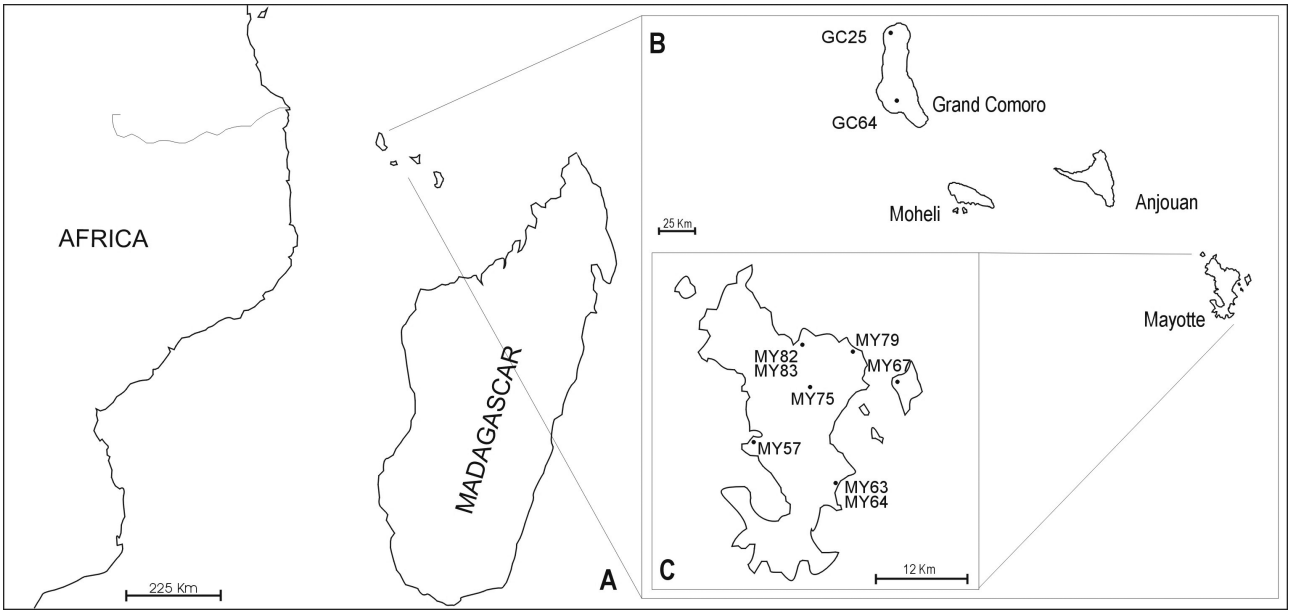

Fig. 1. – Maps showing : A) the position of Comoros archipelago relative to African mainland and Madagascar, B) the Comoros archipelago and the sampling sites in Grand Comoro and C) the sampling localities in Mayotte. Codes are given in Table 1.

INTRODUCTION

The four major islands of the Comoros archipelago lie about 200 Km west of the northern tip of Madagascar, at the entrance of the Mozambique Channel (Fig. 1). After the separation of Madagascar from Mozambique, this volcanic chain of islands was formed, during the Miocene to the Late Pleistocene, and has been colonized by the flora and fauna of both Africa and Madagascar, which had already differentiated. The youngest of the Comoros is Grand Comoro (0.5 My), dominated by the volcano Karthala, which is still active, giving this island a uniform topography. Mayotte is the oldest, with 10-15 My, and harbours several volcanoes, being the result of the union of previously independent massifs. These dates correspond to the estimated age of the volcanic origin of the islands (MONTAGGIONI & NOUGIER, 1981 ; NOUGIER et al., 1986). The age of the oldest exposed lavas is considerably

more recent : 0.13±0.02 My for Grand Comoro and 7.7±1 for Mayotte. These islands never had contact with other landmasses and are separated from each other and from Africa by sea depths of more than 3600 m (EMERICK & DUNCAN, 1982; NOUGIER et al., 1986).

The genus *Furcifer* (Chamaeleonidae : Reptilia) is represented in these islands only by two endemic species; *Furcifer cephalolepis* Günther, 1880, in Grand Comoro and *Furcifer polleni* Peters, 1874, in Mayotte. The extant 14 species of this genus all inhabit Madagascar, with one, *F. pardalis* Cuvier, 1829, also present in Mauritius and Reunion Islands, probably representing another natural oceanic dispersal (RAXWORTHY et al., 2002).

In a previous study involving many Chamaeleonidae species, RAXWORTHY et al. (2002) found support for a Madagascan origin for chameleons with multiple "out-of-Madagascar" dispersal events, one of them being the colonization of the Comoros archipelago by *Furcifer* species. Based only on morphological data, these authors placed *F. cephalolepis* and *F. polleni* as sister-taxa and related to the *F. oustaleti* and *F. lateralis* groups from Madagascar. However, attempts to interpret a morphological phylogenetic tree in terms of colonization sequence are compromised by ecogenetic adaptation to current selective pressures influencing the tree (THORPE et al., 1994). This could be the case here, as they are placed together by two non-unique synapomorphies : both Comoros chameleons have reduced body size and lost lung diverticula, probably as function of body size. Dwarfism in island species is a fairly common evolutionary response, and is presumably adaptive, thus such adaptations may well have evolved in parallel. Therefore, to further assess the position of these species within the *Furcifer* clade on the basis of DNA sequence data, we obtained partial sequences of the ND4 gene, from the same region as RAXWORTHY et al. (2002). We also used ND4 sequences to assess intraspecific diversity within *F. cephalolepis* and *F. polleni.* Being a fast evolving gene, this is an adequate marker to use in recent evolutionary events. Considering the ages of the islands, and assuming just one colonization event, we expected minimal diversity within Grand Comoro, and also between *F. cephalolepis* and its presumed sister-taxa, *F. polleni*. Concerning Mayotte, its age and, in particular, its conglomerate nature, might have led to high genetic diversity within this island, as seen in reptiles from Tenerife, in the Canary Islands (THORPE et al., 1994, 1996; BROWN & PESTANO, 1998).

TABLE 1

Sample code, locality and accession numbers of *Furcifer* specimens used in this study. All other samples were from RAXWORTHY et al. (2002).

Species	Locality	Code	Acession number
Furcifer cephalolepis	Foret de la Guille, Grand Comoro	GC 25	DQ086038
Furcifer cephalolepis	Belvedere, Grand Comoro	GC 64	DQ086039
Furcifer polleni	Sada road, Mayotte	MY 57	DQ086040
Furcifer polleni	Bandrélé	MY 63	DQ086041
Furcifer polleni	Bandrélé	MY 64	DQ086042
Furcifer polleni	Airport, Dzaouzi islet, Mayotte	MY 67	DQ086043
Furcifer polleni	Vahibé	MY 75	DQ086044
Furcifer polleni	Mahicavo	MY 79	DQ086045
Furcifer polleni	Longoni	MY 82	DQ086046
Furcifer polleni	Longoni	MY 83	DQ086047

MATERIALS AND METHODS

Tail tips from eight *F. polleni* and two *F. cephalolepis* were collected in Mayotte and Grand Comoro (geographic locations of the specimens are given in Table 1 and Fig. 1) and genomic DNA was extracted following standard high-salt protocols. A fragment including the terminal portion of the ND4 gene and the tRNA's for Serine, Histamine and Leucine was amplified by PCR using the primers published by ARÉVALO et al. (1994) and sequences from both strands were obtained on an automated sequencer (ABI 310). Alignment was performed using Clustal W 1.6 (THOMPSON et al., 1994; default parameters) and adjusted manually in BioEdit (HALL, 1999). Sequences from other *Furcifer* species from Madagascar and Reunion Island previously published by RAXWORTHY et al. (2002) were also included. *Chamaeleo jacksoni* and *Calumma cucullata* were used as outgroups. Ambiguous alignment regions (12 bp of the tRNA's) were excluded from all analyses. To select the model of nucleotide substitution that better fits our data set, the hierarchical likelihood-ratio test was carried out using Modeltest 3.06 (POSADA & CRANDALL, 1998). Sequences were then imported into PAUP*4.0b10 (SWOFFORD, 2003) and the chosen model used to perform Maximum Likelihood (ML) analysis with random sequence addition (10 replicate heuristic search). Maximum Parsimony (MP) analysis was also carried out with random sequence addition (100 replicate heuristic searches) and support for nodes was estimated through the bootstrap technique (FELSENSTEIN, 1985) with 1000 replicates. Bayesian analysis was implemented using MrBayes v.3.0 (HUELSENBECK & RONQUIST, 2001) with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. All analysis started with randomly generated trees and ran for 10^6 generations, saving one tree in each 10 generations. The log-likelihood values of the sample points were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Combining the remaining trees, a 50% majority rule consensus tree was generated. The frequency of any particular clade of the consensus tree represents the posterior probability of that clade (HUELSENBECK & RONQUIST, 2001). Two independent replicates were conducted and inspected for consistency to check for local optima (HUELSENBECK & BOLLBACK, 2001). To assess variation within *Furcifer polleni* (from Mayotte), these sequences

(total length of 807 bp) were joined into a median network (BANDELT et al., 2000).

RESULTS

Ten sequences were obtained and 22 sequences, representing 14 taxa, were included in the analyses, for an aligned length of 815 bp. The most appropriate model of evolution for this dataset was the GTR, with an estimate of invariable sites (0.4116) and a discrete approximation of the gamma distribution (1.1168). ML, MP and Bayesian analyses gave congruent estimates of relationships, with ML and Bayesian trees having identical topologies, and the MP tree having one difference in topology relative to these (Fig. 2). Concerning the intraspecific diversity, the two individuals from *F. cephalolepis* had distinct haplotypes, presenting 0.87% divergence (seven differences in 807 bp). Within *F. polleni*, five distinct haplotypes were found in a total of 8 individuals, with a maximum divergence of 0.74% (six differences in 807 bp) and without any clear geographic structure (Fig. 3).

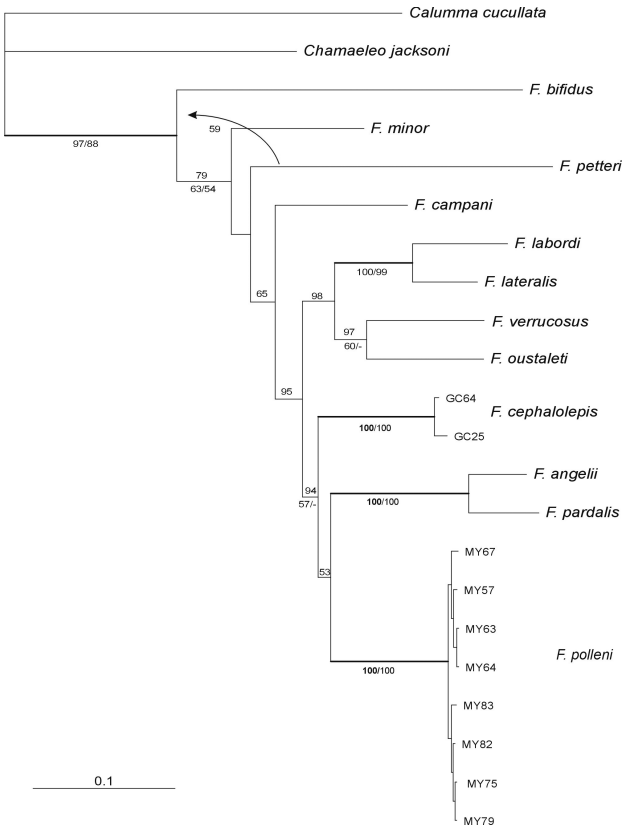

Fig. 2. – Tree derived from the Bayesian analysis of the 815 bp of the ND4 gene. Posterior probabilities are given above the branches with the bold branches having the value of 100%. Below the branches, bootstrap values for ML and MP are indicated (ML/MP). For both analyses, only bootstrap values above 50% are represented. The arrow indicates a variation in the position of one branch in the MP analysis and the respective bootstrap value is indicated below.

DISCUSSION

Both *F. cephalolepis* and *F. polleni* represent distinct and very well supported branches. Their relative position and long branch lengths show that they are probably not sister-taxa, as previously suggested. Indeed, independently of the method used in the analyses, *F. polleni* always appeared as sister-taxa of the *F. angeli* and *F. pardalis* group from Madagascar, with *F. cephalolepis* splitting first from their common ancestor. This, points to independent colonization of both Comoro Islands. While we cannot exclude alternative hypotheses, like the existence of a very divergent unsampled lineage in Mayotte that could be the "sister-group" of *F. cephalolepis* from Grand Comoro, these are less likely due to our geographically widespread sampling in Mayotte. Clearly, further sampling is needed, especially from the extant *Furcifer* species from Madagascar (eight species from this genus are not included in this analysis), to clarify the relationships between them, and to better understand the process of colonization of the Comoros Islands.

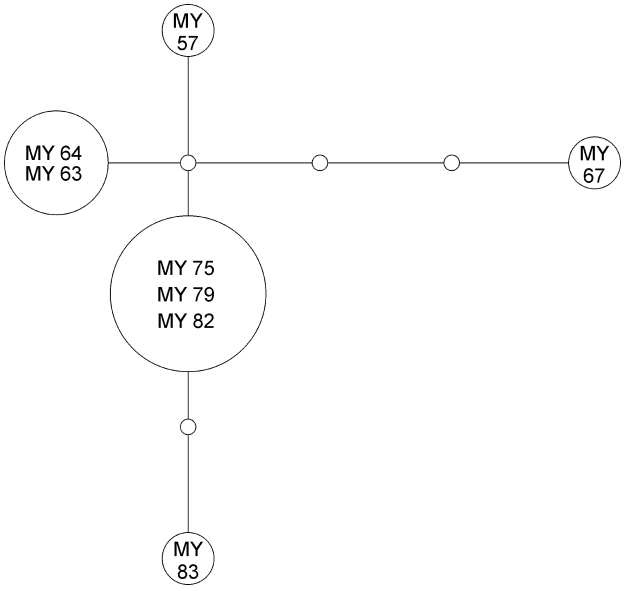

Fig. 3. – Network showing relationships between *Furcifer polleni* haplotypes found in Mayotte. Diameter of circles is proportional to the frequency of each haplotype and empty circles represent missing haplotypes.

The genetic divergence found inside *Furcifer cephalolepis* was surprisingly high, considering the age of the island. The two haplotypes from Grand Comoro have 0.87% genetic divergence (uncorrected p-distance), which, according to the vertebrate ectotherm mtDNA divergence rate – often used for this gene – of 0.4-0.6% per lineage per million year (RAND, 1994; CACCONE et al., 1999), would correspond to 0.73 to 1.1 million years divergence.

Even using a broader interval of sequence variation : 0.25-0.7% per lineage per million year (AVISE et al., 1992; CACCONE et al., 1997) we obtain estimates of divergence between 0.63 and 1.76 My, always higher than the oldest estimates for the age of the island – 0.5 My. So, the ND4 gene in *Furcifer* seems to be evolving faster that the rates generally used (at least at a value of 0.88% per lineage per million years if we use 0.5 My – age of Grand Comoro – as a calibration point. Moreover, these are minimum estimates of the divergence; based only in two individuals, as with more individuals even more divergent haplotypes could be found.

One possible explanation is that ND4 gene in *Furcifer* is evolving faster than predicted by these molecular clocks. However an alternative explanation for this result is that divergence within *F. cephalolepis* predates the colonization of Grand Comoro and this colonization was made by individuals including already differentiated mtDNA lineages.

Islands with known geological ages are often thought to be ideal for calibrating molecular clocks (CARRANZA et al., 2000) and the common procedure is that when sister-taxa are found on neighbouring islands to assume that the age of the younger island represents an approximate estimate for the maximum age of the split between the "offspring" population on the younger island and the "parental" population on the older island. However our results suggest that "universal" clocks are extremely inaccurate. Furthermore, precise phylogenies are needed – if divergence values between *F. polleni* and *F. cephalolepis* (assuming incorrectly they were sister taxa) were compared to the age of Grand Comoro we would obtain an erroneous estimated rate of evolution of at least 11% per lineage per million year. This type of calculation, focusing on the observed divergence between islands and using the age of the youngest one as a calibration point, is still commonly used (e.g. BROWN & PESTANO, 1998; WARREN et al., 2003). Our results highlight the importance of also assessing within-island diversity when estimating divergence rates.

In conclusion, our results suggest that the Comoros were independently colonized twice by *Furcifer* from Madagascar. They also suggest that this region of the ND4 gene and associated tRNA's may be evolving faster than that predicted by ectothermal vertebrate molecular clocks, which has implications for the estimated times of colonization of other island groups by chameleons. This further highlights the inaccuracies of generalized applications of molecular clocks, even when calibrated using known geological values such as the age of islands. The fast rate of evolution of this region of mtDNA makes it highly suitable for phylogeographic studies.

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Distribution patterns and indicator species of butterfly assemblages of wet meadows in southern Belgium

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ABSTRACT. Focal species are a valuable tool for proposing and evaluating management practices for biodiversity conservation. Assemblages of indicator species could be used to cover a wide range of habitats. We identified the main patterns of variation in butterfly assemblages on a diverse set of wet meadows in southern Belgium. We used multivariate techniques to identify the butterfly assemblages and the species that characterize these habitats. Three main assemblages were identified, based principally on the dominance of five butterfly species : *Brenthis ino, Clossiana selene, Lycaena helle, Lycaena hippothoe* and *Proclossiana eunomia*. These are indicator species of different habitats structured along a vegetation gradient. This gradient is partially determined by altitude and edaphic factors (base-richness, pH, fertility). We assume that focusing the conservation practices upon these species will promote the preservation of a wide range of organisms inhabiting the wet meadows.

KEY WORDS : butterflies, indicator species, multivariate analysis, wet meadows, biodiversity conservation.

INTRODUCTION

Almost every ecosystem on Earth is going through a strong anthropogenically induced disturbance biodiversity crisis, in which several thousands species are involved. Natural and semi-natural biotopes are suffering from high rates of modification and, as a consequence, the number of species threatened by extinction increases. In order to evaluate and monitor biodiversity as well as to implement conservation measures, the development and use of shortcuts is a necessity. Obviously, it is not viable to consider the development of a detailed study for each one of the endangered species. One possible approach is based on diversity measures, but it has several limitations like excluding particular taxonomic information about the communities (NOSS, 1990; DUFRÊNE & LEGENDRE, 1997). The criterion of representative diversity which is based on the recognition of assemblages of species that are typical for specific habitats seems more interesting (DUFRÊNE & LEGENDRE, 1997). A potentially useful tool is the single-species approach based on focal or surrogate species, which can indicate ecological change, patterns of richness or habitat type (NOSS, 1990; CARO & O'DOHERTY, 1999; FLEISHMAN et al., 2000). Focal species include umbrella, flagship and different kind of indicator species (MCGEOCH, 1998; SIMBERLOFF, 1998). Some of these species could be used as targets for conservation efforts, assuming their preservation may help to protect other species that share the same habitats. Thus, the use of surrogate species may be a valuable tool for conservation planning, allowing considerable reductions of time and funding costs. Moreover, the use of assemblages instead of single species as indicators enables the enlargement of the focus of the single-species approach to wider ecological situations (KREMEN, 1992). Assemblages of species, with determined species richness, level of dominance, and taxonomic composition, can characterize different habitats. The use of a small set of surrogate species allows the inclusion of a broader range of habitats. Using keystone species may be an interesting alternative to surrogate species (SIMBERLOFF, 1998). However, the effort and time demanded by this approach in the study of the community processes and the high rate of degradation of the biotic diversity, makes the use of other tools necessary (e.g., surrogate species) enabling conservation managers to develop faster responses to the anthropogenic disturbances. Hence, important research effort is needed to select appropriate surrogate species.

Lepidoptera have been proposed as surrogate species by several authors (KREMEN, 1992; BECCALONI & GASTON, 1995; FLEISHMAN et al., 2000). Several features of the butterflies make them good candidates for indicator, umbrella and/or flagship species (NEW, 1997; FLEISHMAN et al., 2000; MAES & VAN DYCK, 2001). They have a wide distribution, are relatively easy to sample and identify, and both as individuals and as species they show important numbers in different ecosystems (BLAIR, 1999; CARO & O'DOHERTY, 1999; RICKETTS et al., 2002). They are also strongly influenced by local weather and highly sensitive to environmental changes (SPITZER et al., 1997), besides being charismatic insects that could attract the public attention. Finally, some authors have identified patterns of co-variation between the abundance and/or the richness of Lepidoptera and those of other taxonomic groups (BLAIR, 1999; SWENGEL & SWENGEL, 1999). How-

ever, these relationships are highly dependent on the taxa and the spatial scales considered (RICKETTS et al., 2002).

Butterflies are extremely sensitive to changes in vegetation composition and structure, and different types of vegetation show different butterfly species composition. So, the butterfly assemblages may be used to characterize different habitats (ERHARDT, 1985). Plants are the essential source of nourishment of butterflies; some specific plant species provide the trophic resources for caterpillars, while others provide nectar for adults. The vegetation can also play an important role for butterfly survival offering particular structural elements for sun-basking or mating and determining certain suitable microclimates (DOVER et al., 1997). Therefore, it would be expected that butterflies respond more strongly to vegetation gradients than to edaphic gradients (SAWCHIK et al., 2003).

Human disturbance in Western Europe, including Belgium, has accelerated during the second half of the 20th century, resulting in a very high pressure on some particular ecosystems. An important factor in this relatively recent panorama has been changes in land use, i.e. the abandonment of traditional agricultural practices (MAES & VAN DYCK, 2001). Some of the most threatened ecosystems are the semi-natural wet meadows, which have been abandoned or modified by modern and intensive agricultural practices. Many of these biotopes are being invaded by shrubs or trees, while others suffer eutrophication, pollution or acidification. Moreover, these modifications lead to an increasing isolation of the remaining fragments. The result is that semi-natural humid grasslands are seriously threatened in Belgium (GOFFART et al., 2000).

The objectives of this study were : (1) to describe the main variation pattern in the butterfly fauna of a wide range of wet meadows; (2) to identify the indicator species of the different habitat types; (3) to explore the relationships between the butterfly assemblages and the vegetation and the soil properties of the sites.

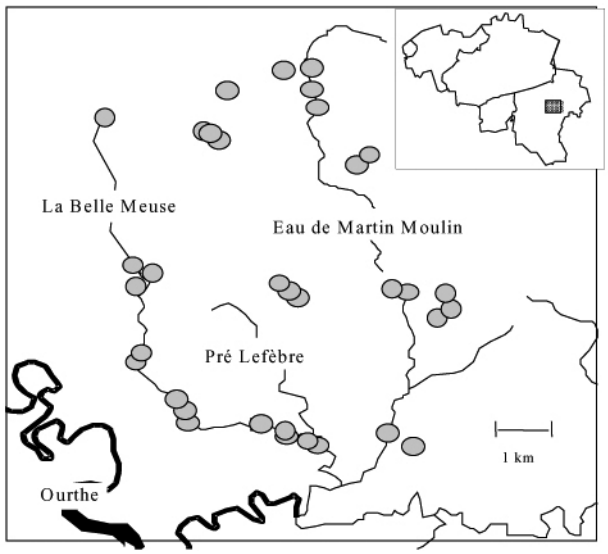

Fig. 1. – Study area in the Plateau des Tailles, Ardennes, Belgium.

METHODS

Study area

The studied area is the basin of the Martin-Moulin river located in the Plateau de Tailles (Fig. 1). This high plateau (max. height 654 m) is situated in the Ardennes biogeographical area, southern Belgium. Coniferous forests and pastures dominate the landscape. Immersed in this mixed forest-prairie matrix there are several semi-natural habitats, particularly a great diversity of wet meadows and mires. These meadows are biologically interesting habitats, displaying high levels of species richness and sustaining populations of many vulnerable species. Thirty-four sites were selected for the present work, all of them wet meadows. They account for all the main vegetation associations that may be found in this area.

Field measurements

The definition of the sites was based on homogeneous floristic composition. All these sites show relatively well-defined boundaries. We identified all the plant species present in a quadrate of 5 (5 meters randomly placed in each site. The size of the quadrate is considered convenient to describe meadow communities (KENT & COKER, 1992). Then, the size of the quadrate was progressively increased until no new species were identified. Percentage cover of plants was estimated visually. These vegetation surveys were made during the summer of 1996.

Within each site, soil samples were collected at five randomly located places. The soil was sampled at 0-20 cm depth. The samples were pooled, and the following edaphic variables measured : soil pH, organic matter (OM), soil moisture (SM), total N, concentrations of Ca, K, Mg and Na. We also determined water-table depth (WT) recorded in 3 locations at each site in the summer of 1997. Values of WT were averaged prior to data analysis. As OM and total N were highly correlated ($r > 0.9$) we dropped N from the analysis data set. The chemical analyses were carried out at "l'Unité d'Écologie des Prairies" (UCL/AGRO/ECOP/Belgium).

Butterflies (Rhopalocera : Papilionoidea) were recorded by visual observation along zigzag transects from May to August of the years 1994, 1995 and 1996. More precisely, in each site we walked at a regular step until the total area was visited. We registered all the individuals observed in a corridor of approximately 6 m of width. We sampled each site as many times as possible in sunny conditions (POLLARD, 1977; THOMAS, 1983). The sampling program covered the flying periods of most species and ensured that a minimum of 3 sampling visits was made for each species in each site and each year.

As a measure of relative abundance of the butterfly species we used the maximum number of observed individuals in each site (SMALLIDGE et al., 1996). This measure allows controlling sampling intensity and weather differences. Moreover, field experience in such habitats demonstrated that after three or four visits well distributed along the main activity period, the maximum does not change considerably. Indeed, very similar results were obtained with additional analyses using other measures of relative abundance (e.g. mean, median, and sum). The

data from the 3 years were pooled, so the maximum over the 3 years was used as a measure of abundance. Because all the sites were small (range from 0.1 to 2 ha; median = 0.775), they might be highly affected by stochastic effects. By pooling the data from different years, we integrate over annual weather effects on local abundance as well as extinction and rescue events generated by metapopulation dynamics on a network of habitat patches (HANSKI et al., 1995; FLEISHMAN et al., 1999).

Assemblage structure and indicator species identification

The structure of the butterfly species assemblage was studied by ordination and classification methods. The butterfly matrix details the distribution of 15 species in 34 sites and shows a 31% of zeros. As suggested by LEGENDRE & LEGENDRE (1998), rare species (< 1% of the total number recorded) were removed from the analysis. In general, these rare species were represented by single, vagrant individuals that probably do not concern local populations. This category also concerns migratory species as *Vanessa atalanta* and *Cynthia cardui*. Moreover, correspondence Analysis (CA) and classification techniques are sensitive to the occurrence of rare species, so these species may distort the analysis impeding a reliable description of the main patterns of variation (EZCURRA, 1987). A distance matrix was constructed using the Bray Curtis coefficient. Hierarchical agglomerative clustering was conducted on the distance matrix using the UPGMA method. We used the IndVal approach developed by DUFRÊNE & LEGENDRE (1997) to identify the indicator species of a site typology resulting from a clustering of the butterfly data set. For each step of the clustering process indicator species are revealed by an index (IV, i.e., Indicator Value) that combines an estimator of the species specificity and fidelity (MCGEOCH & CHOWN, 1998). With this kind of approach the indicator species are defined as the most characteristic species of a site cluster, found mostly in a site cluster of the typology (specificity) and present in the majority of the sites belonging to that cluster (fidelity). The significance of the IV index is assessed by a permutation approach (1000 permutations). Species with significant high IndVal values (> 70%) show strong habitat fidelity and specificity, so they may be considered as characteristic species of the assemblages (MCGEOCH et al., 2002.).

Ordination diagrams can be used to select a subset of the species as indicators (KREMEN, 1992). In order to identify the main information axes, ordination was carried out performing CA. Because CA ordination scores represent approximately optima localization of the species in the ordination plan (TER BRAAK & PRENTICE, 1988), the diagram could be used to find assemblages of characteristic species along the gradients (KREMEN, 1992).

Butterflies-habitat relationships

A first approach to interpret the ordination results with the aid of external data was to compute Spearman correlation coefficients (r_S) between CA ordination scores from the butterfly matrix and altitude (m). Additionally, we computed correlation coefficients between the scores of the CA axes and the diversity of the butterfly assemblages. In each site we computed species richness (S), Shannon diversity (H') and evenness (E). The Simpson's index (D) was used as a measure of dominance. All of these measures are widely used in ecology literature (KREBS, 1989; MAGURRAN, 1988).

In order to explore the relationships between the butterfly fauna and the habitat, defined by the edaphic factors and the plant assemblages, we used CO-inertia Analysis (COA). The choice of COA instead of canonical correspondence analysis (CCA) was based on the high number of variables compared to the number of sites. When the number of variables equals the number of sites CCA becomes a CA (TER BRAAK & PRENTICE, 1988; JONGMAN et al., 1995). COA is a symmetric ordination technique well adjusted to analyze data sets with many explaining variables and a relatively low number of sites (DOLÉDEC & CHESSEL, 1994). It is a symmetric method because it does not assume that one matrix is constituted by explaining variables and the other by explained variables. Indeed its use is well justified in this study because we are not attempting to explain the abundance of the butterfly species as a function of plant assemblages. We are merely interested in the associations between plants and butterflies without assuming a causal relationship between them. In a first step, faunal and environmental matrices are analyzed by standard ordination methods as CA or PCA. In a second step, COA maximizes the covariance between the first axes of variation of the two matrices.

We performed two COA's to link the CA ordination of butterfly data to vegetation and edaphic data. The first analysis (COA_1) linked the butterfly ordination to an ordination obtained by CA performed on the vegetation frequency data set. Because there was a high number of plant species recorded, and the fact that the great majority among them showed very low frequencies and cover values, we decided to remove rare species from the data set to gain in clarity and computation efficiency, and to reduce noise and redundancy. The vegetation matrix details the distribution of 40 species and shows 53% of zeros. The second analysis (COA_2) linked the butterfly ordination to a PCA performed on the correlation matrix of the edaphic data. The edaphic variables were log transformed prior to PCA. The signification of the co-inertia values was tested by a permutation procedure (1 000 permutations). Additionally, we performed another co-inertia analysis, COA_3, to explore the relationships between vegetation and edaphic factors.

CA was performed with the CANOCO software (TER BRAAK & SMILAUER, 1998). We used the R package (CASGRAIN & LEGENDRE, 1999) to compute correlation and distance coefficients and to perform cluster analysis. The version 2.0 of the program IndVal (DUFRÊNE & LEGENDRE, 1997) was used to identify the indicator species. COA was performed with ADE-4 (THIOULOUSE et al., 1997).

RESULTS

We identified a total of 30 butterfly species but only 15 species were retained for most of the analyses (Table 1). The matrix of butterfly abundance used in subsequent analyses is presented in Table 2. Hesperoidea were mostly represented by two species : *Thymelicus lineolus* and *T.*

sylvestris. For practical reasons, *Thymelicus* species were not retained for the analysis. These are two relatively common species that are not easily distinguishable in the field.

TABLE 1

Number of individuals, number of occupied sites and butterflies codes (used as labels in the figures).

Code	Species	No. individuals	No. sites
AGL	*Aglais urticae* (Linnaeus, 1758)	176	27
ANT	*Anthocaris cardamines* (Linnaeus, 1758)	50	21
APH	*Aphantopus hyperantus* (Linnaeus, 1758)	1,036	34
APO	*Aporia crataegi* (Linnaeus, 1758)	56	20
ARA	*Araschnia levana* (Linnaeus, 1758)	39	19
BRE	*Brenthis ino* (Rottemburg, 1775)	799	26
CLO	*Clossiana selene* ([Denis & Schiffermüller], 1775)	194	17
GON	*Gonepteryx rhamni* (Linnaeus, 1758)	39	21
INA	*Inachis io* (Linnaeus, 1758)	41	23
LHE	*Lycaena helle* ([Denis & Schiffermüller], 1775)	243	22
LHY	*Lycaena hippothoe* (Linnaeus, 1761)	102	18
MAN	*Maniola jurtina* (Linnaeus, 1758)	251	32
PNA	*Pieris napi* (Linnaeus, 1758)	120	29
PRA	*Pieris rapae* (Linnaeus, 1758)	29	20
PRO	*Proclossiana eunomia* (Esper, 1799)	221	22
	Argynnis aglaja (Linnaeus, 1758)	2	2
	Boloria aquilonaris (Stichel, 1908)	20	1
	Callophrys rubi (Linnaeus, 1758)	15	10
	Coenonympha arcania (Linnaeus, 1761)	5	4
	Coenonympha pamphilus (Linnaeus, 1758)	10	4
	Cynthia cardui (Linnaeus, 1758)	24	16
	Erebia medusa ([Denis & Schiffermüller], 1775)	4	4
	Lasiommata megera (Linnaeus, 1767)	2	2
	Melitaea diamina (Lang, 1789)	32	12
	Mellicta athalia (Rottenburg, 1775)	6	5
	Papilio machaon (Linnaeus, 1758)	2	2
	Pieris brassicae (Linnaeus, 1758)	15	11
	Polygonia c-album (Linnaeus, 1758)	3	1
	Polyommatus icarus (Rottenburg, 1775)	6	6
	Vanessa atalanta (Linnaeus, 1758)	14	12

TABLE 2

Matrix of butterfly abundance, recorded as the maximum number of individuals observed in each site.

AGL	ANT	APH	APO	ARA	BRE	CLO	GON	INA	LHE	LHI	MAN	PNA	PRA	PRO
6	0	15	5	2	28	0	3	1	9	2	6	5	1	3
4	0	17	0	2	28	0	1	0	0	0	3	2	0	0
6	1	42	2	0	54	0	1	1	15	0	13	3	1	6
0	0	53	1	1	84	0	0	1	7	0	14	2	2	15
3	0	14	0	1	42	0	1	1	6	0	7	1	1	2
20	1	32	10	0	13	0	0	3	25	2	24	0	0	12
0	2	20	2	3	30	0	0	2	25	0	4	4	1	8
19	2	40	1	2	58	0	1	2	3	0	23	2	1	18
2	5	40	2	4	75	0	0	2	0	0	20	7	2	0
0	0	15	0	1	10	0	2	3	7	0	0	3	1	8
5	0	25	3	0	6	3	1	0	1	1	10	2	0	2
4	1	25	7	3	19	1	5	2	8	4	12	3	0	2
2	1	20	3	2	2	4	3	2	25	2	5	2	1	17
5	0	30	0	0	0	21	1	1	0	7	3	13	2	0
13	0	36	2	0	37	0	0	1	4	0	7	2	0	0
15	5	35	3	6	10	0	2	7	16	0	6	7	1	10
2	4	29	1	1	5	1	2	0	0	0	5	5	1	0
3	2	30	4	1	0	20	5	2	1	0	4	5	0	5
0	1	30	1	0	20	0	0	0	2	0	5	1	2	3
1	0	40	1	0	0	9	1	0	0	0	8	0	0	0
15	5	30	3	0	17	5	2	1	5	8	10	7	2	10
15	5	20	2	1	8	10	0	0	2	7	6	7	1	8
5	1	50	0	0	30	4	0	0	0	10	5	1	0	0
1	2	46	0	2	2	2	1	2	9	6	5	3	1	7
0	3	33	0	2	0	6	0	1	29	4	1	4	1	35
3	2	32	0	0	0	27	0	0	0	13	6	7	0	0
1	0	15	0	0	0	53	1	1	0	10	0	1	3	0
2	0	25	1	0	10	0	1	2	38	0	2	2	0	12
11	0	64	2	1	92	0	3	1	0	1	12	0	0	0
7	0	43	0	0	0	1	0	0	0	2	8	0	0	5
0	1	30	0	2	25	0	1	1	5	3	4	4	0	17
1	2	20	0	2	74	0	0	0	0	0	4	10	0	0
0	1	10	0	0	0	17	0	0	0	5	2	0	1	0
5	3	30	0	0	20	10	1	1	1	15	7	5	3	16

We recorded a total of 138 plant species, but the great majority of them were only present in low abundance and frequency species. The 25 species considered for multivariate analysis are listed in Table 3.

TABLE 3

Number of occupied sites and plant codes (used as labels in the figures).

Code	Species	No. Sites
ACN	*Agrostis canina*	21
ACP	*Agrostis capillaris*	4
ANG	*Angelica sylvestris*	27
CNI	*Carex nigra*	10
CRO	*Carex rostrata*	5
CIR	*Cirsium palustre*	32
DES	*Deschampsia caespitosa*	21
FES	*Festuca rubra*	14
FIL	*Filipendula ulmaria*	24
GPA	*Galium palustris*	24
GTE	*Galeopsis tetrahit*	17
HLA	*Holcus lanatus*	25
HMO	*Holcus mollis*	6
JAC	*Juncus acutiflorus*	25
JEF	*Juncus effusus*	20
LOT	*Lotus pedunculatus*	25
LYS	*Lysimachia vulgaris*	13
POL	*Polygonum bistorta*	29
POA	*Poa trivialis*	18
RAN	*Ranunculus repens*	17
RUM	*Rumex acetosa*	24
SCI	*Scirpus sylvaticus*	14
URT	*Urtica dioica*	14
VAL	*Valeriana repens*	17
VIO	*Viola palustris*	18

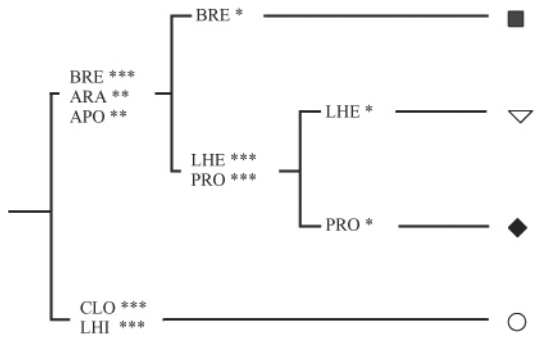

Fig. 2. – UPGMA clustering results and the indicator species proposed by the IndVal approach (IV). ***. Maximal and significant (at the 0.05 level), IV > 70%. **. Maximal and significant (at the 0.05 level), IV < 70%. *. Significant (at the 0.05 level) but not maximal IV.

The site typology obtained by UPGMA (Fig. 2) was very similar to that obtained using a *k*-means non-hierarchical partition method (SAWCHIK, 1999) so we concluded that the hierarchical pattern of the groups was robust (DUFRÊNE & LEGENDRE, 1997). First, the whole set of sites was split in two main groups separating lowland meadow sites from high altitude, acid and poor (oligotrophic) sites. At this step, IndVal identified only *B. ino* (IV = 89.7), *A. levana* (IV = 69.0) and *A. crataegi* (IV = 65.5) as having significant IndVal values for the group constituted by all the lowland meadow sites. The oligotrophic sites, represented by peat-bogs (rich in *Carex sp.* and *Sphagnum sp.*) and wet rush (*Juncus sp.*) meadows, were characterized by *C. selene* (IV = 90.2) and *L. hippothoe* (IV = 76.8). The next level subdivided lowland meadows in one cluster of eutrophic sites and another cluster of mesotrophic meadows dominated by the association *Deschampsio cespitosae-Polygonetum bistortae*. The wet meadowsweet (*Filipendula ulmaria*) grasslands are distributed between these two clusters. Two butterfly species showed statistically significant IndVal values : *L. helle* (IV = 85.8) and *P. eunomia* (IV = 82.5). Only *B. ino* showed a significant, although not maximal, indicator value (IV = 71.0) for the cluster of eutrophic sites. Finally, the classification divides the cluster of bistort meadows in one group characterized by *L. helle* (53.4) and another group characterized by *P. eunomia* (48.4). These IV values were significant but not maximal, so the division at this level is less defined. This suggests subtle differences in the gradient of humid bistort grasslands from rush meadows to meadowsweet grasslands. After this division level the sum of IndVal values decreased, suggesting that the next divisions of the typology are not informative.

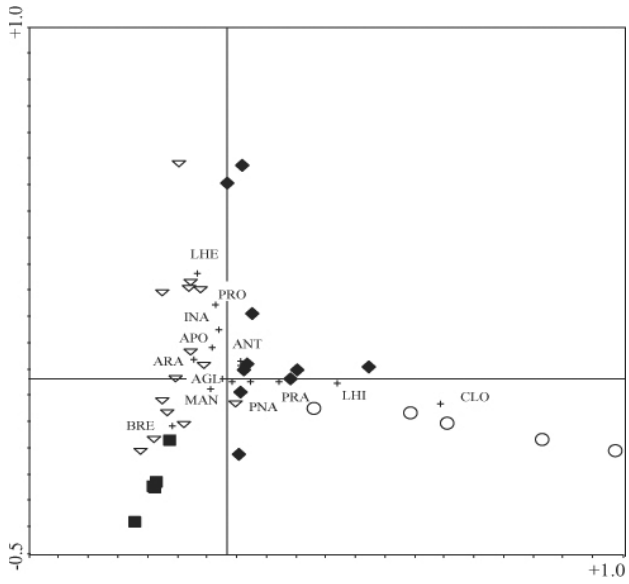

Fig. 3. – Ordination plot (2 first axes) of the Correspondence Analysis (CA) of the butterfly species matrix. The symbols correspond to the groups identified by the UPGMA algorithm.

The total variance explained by the first two CA axes was 64% (40.4% and 23.6% respectively). The two-dimension diagram obtained from CA ordination (Fig. 3) revealed the same species assemblage structure suggested by the clustering results. The species that mainly contribute to the species assemblage originality were *B. ino*, *C. selene*, *Inachis io, L. helle*, *L. hippothoe*, and *P. eunomia*. The first axis opposes *C. selene* to *B. ino*. *Clossiana selene* was present in high altitude, oligotrophic sites while *B. ino* was associated with lowland meadows. The second axis showed a rather slight arch effect, suggesting the prevalence of the main gradient. However, it is unlikely that this axis was simply an artifact and probably reflects a secondary gradient. This was confirmed by detrended correspondence analysis and principal coordinate analysis two-dimension ordinations (not shown),

which produced essentially the same spatial arrangement of the data revealed by CA.

The second axis opposes bistort meadows characterized by *L. helle* and *P. eunomia* to meadowsweet grasslands sites strongly associated with *B. ino*. The eutrophic sites dominated by *F. ulmaria* and *Urtica dioica* are located at one extreme of this axis, while the other meadow sites are characterized by the association of *Deschampsia cespitosa* and *Polygonum bistorta*. However, the slight arch effect suggested by this axis could also be interpreted in another way. The species repelled to the extreme of the parabola (*L. helle, P. eunomia*) would be typical of the middle of the gradient, whereas the species in the center of the parabola would be more widespread, generalist or ubiquitous species (LEBRETON & YOCCOZ, 1987; DUFRÊNE & LEGENDRE, 1997). No significant correlations were observed between species' frequencies among sites and species' CA scores. Therefore, we concluded that ordination results did not result from a sampling artifact (KREMEN, 1992). The first eigenvalues were different in magnitude so we concluded that the ordination was stable (OKSANEN & MINCHIN, 1997). The first CA axis showed a statistically significant correlation with altitude (r_S = 0.668). The second axis showed a significantly positive correlation with butterfly diversity (r_S = 0.589). In spite of the fact that species richness, H' and E may be affected by sampling effort variations, in our case, however, the differences do not seem strong enough to have a great impact on these measures. This assertion is supported by the very low correlations showed between the diversity measures, the total number of individuals, and the area of the sites (Table 4).

TABLE 4

Rank correlation computed as Spearman coefficients between the site scores on the two first axis of the correspondence analysis, the patch area and the total number of individuals (N), and butterfly diversity (S = species richness; H' = Shannon index; E = evenness; D = Simpson index). *. Correlation is significant at the 0.05 level. **. Correlation is significant at the 0.01 level.

	Axis 1	Axis 2	Area	N
S	-0,117	+0.427 *	-0,103	+0,136
H'	+0,129	+0.589 **	-0,002	-0,053
E	+0,382	+0.524 **	+0,087	-0,160
D	-0,134	-0.619 **	-0,016	-0,092

TABLE 5

Summary of the co-inertia analyses : inertia and co-inertia values for the butterfly and environmental (CO1 : vegetation; CO2 : edaphic) matrices, and correlation values between the butterfly and environmental ordination axes.

Analysis	Axis	Correlation	Inertia butterflies	Inertia environmental	Co-inertia butterlies	Co-inertia environmental
CO1	1	0,809	0,396	0,503	0,384	0,416
	2	0,818	0,215	0,413	0,204	0,305
CO2	1	0,631	0,396	3,551	0,355	1,944
	2	0,496	0,215	2,091	0,193	2,879

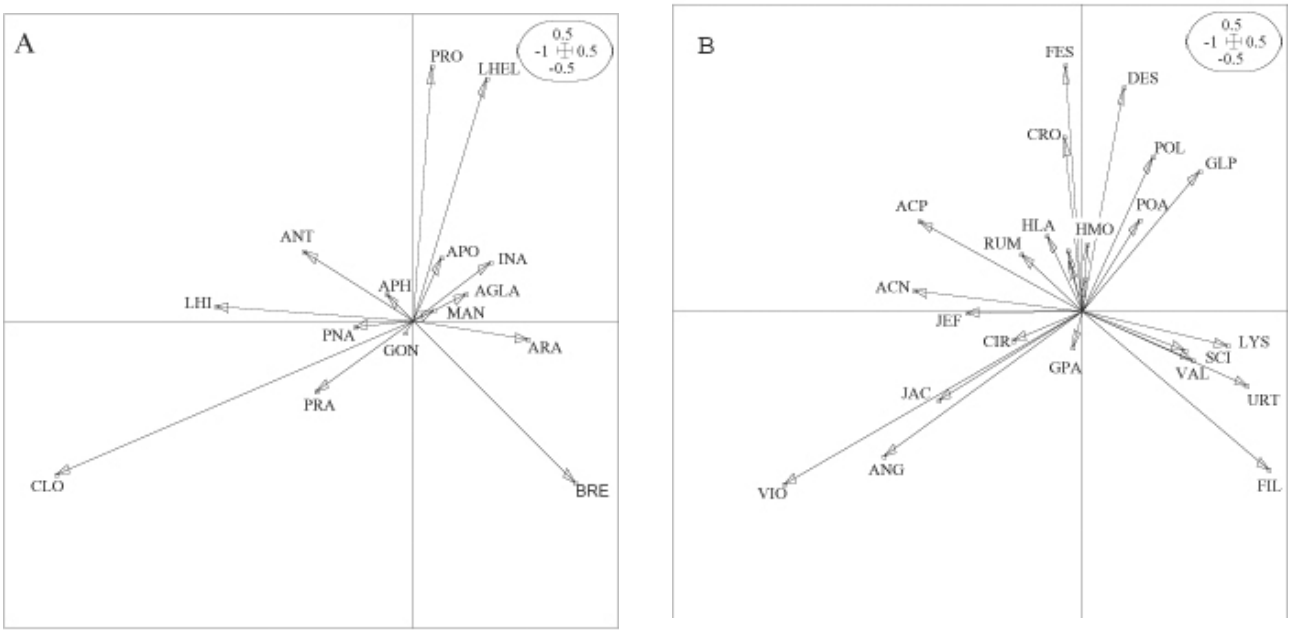

Fig. 4. – Results of the co-inertia analysis performed on the 15 butterfly species and the 25 plant species. (a) Position of butterfly species on the F1 x F2 co-inertia plane. (b) Position of plant species on the F1 x F2 co-inertia plane.

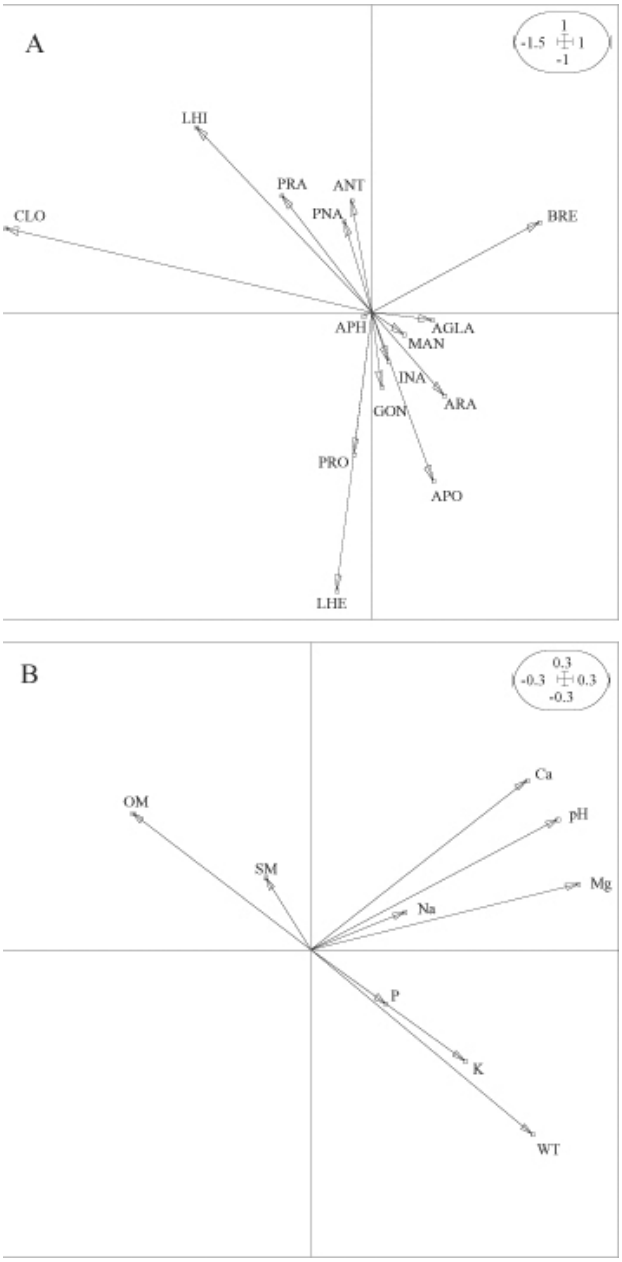

Fig. 5. – Results of the co-inertia analysis performed on the 15 butterfly species and the 9 edaphic variables. (a) Position of butterfly species on the F1 x F2 co-inertia plane. (b) Position of edaphic variables on the F1 x F2 co-inertia plane.

The main two axes of COA explain 55.81% and 22.14% of the common structure shared by the butterfly and the plant matrices. The co-inertia test was highly significant ($p < 0.001$). The inertia values for the two first axes of the CA, both for butterfly and plant species, are shown in Table 5. The correlation values between the butterfly and the plant ordination axes as well as the co-inertia values indicate that the structure described separately for butterfly and plant species data are relatively well captured by the first co-inertia plane. The ordination diagram (Fig. 4) shows almost the same pattern than the CA diagram, indicating that the structure of co-variation between butterfly and plant species succeeds in explaining the main pattern of the distribution of the butterfly assemblages. At one extreme of the first axis we found *C. selene* associated with *V. palustris, A. sylvestris* and *J. acutiflorus*. *Lycaena hippothoe* was also located on this side of the diagram, mainly associated with *A. canina, J. effusus* and *A. capillaris. Brenthis ino* and *A. levana* were situated at the other end of this axis, associated to *F. ulmaria, U. dioica, V. repens* and *L. vulgaris.* In the middle of the axis, *L. helle* and *P. eunomia* occupied sites dominated by *D. cespitosa, F. rubra* and *P. bistorta*. In this study, COA succeeds in removing a strong arch effect seen on the main plane of the CA performed on the vegetation matrix (not shown).

The two main axes of the COA_2 explain 58.56% and 29.12% of the common structure shared by the butterfly and the edaphic matrices. The inertia values for edaphic factors were 3.551 and 2.091 respectively. The co-inertia test was significant ($p = 0.022$). The correlation values between the butterfly and the soil ordination axes as well as the co-inertia values are shown in Table 5. In the ordination diagram (Fig. 5), we visualize that *C. selene* and *L. hippothoe* were associated with acid and wet sites with accumulated OM and low levels of mineral bases. The butterfly species *A. crataegi* and *A. levana* were associated with the sites with the highest levels of K and P which are also less humid and with deeper WT than the previous sites cited. The less acidic sites with the highest levels in Ca and Mg were associated to *B. ino*, while *L. helle* and *P. eunomia* were preferentially situated in relatively dry sites with low levels in bases and OM. The ordination of edaphic factors obtained from COA_3 (not shown) showed a spatial configuration very similar to the ordination obtained from COA_2.

DISCUSSION

Methodological considerations

Multivariate techniques are highly useful tools for identifying sets of indicator species for different habitats along a gradient. Classical and canonical ordination methods allow the study of the relationships between species distributions and environmental gradients. Classification techniques are helpful for identifying discontinuities and to define the main habitat types. In particular, UPGMA combined with IndVal may provide a better alternative than TWINSPAN for classification and identification of indicator species (Dufrêne & Legendre, 1997; Lee & McDonald, 1993). In short, these methods reflect local patterns of habitat heterogeneity indicating which assemblage could be considered as an appropriate indicator of these habitat types (Kremen, 1992).

COA is a useful and robust tool for displaying species-habitat relationships because it allows the simultaneous and symmetric study of two data sets. The symmetric approach allows the connections to be made between tables with different number of environmental variables, taxa and/or sites. As an ordination technique, it also has other potential benefits, for example the removal of the arch effect (Dolédec & Chessel, 1994). This approach may be also useful for defining and comparing biological assemblages indicative of different habitats or to evaluate the effects of disturbances on the habitats.

Vegetation gradients

Complex vegetation gradients partially reflect underlying edaphic gradients, i.e., nutrients, acidity and moisture gradients (OOSTERMEIJER & VAN SWAAY, 1998). The main direction of variation in edaphic factors is probably along the base-richness and acidity gradient. At one extreme of the gradient are located the acid, base-poor sites, at the other those rich in bases and showing neutral pH. This gradient may be very important in structuring the floristic composition of the sites (WHEELER & PROCTOR, 2000; ØKLAND et al., 2001), and hence in determining the composition of the butterfly fauna of these sites.

The analyses of edaphic factors show that two other gradients may be also of great relevance to the composition of the vegetation and fauna. One of these ecoclines is the fertility gradient, determined by the availability of P and K. This gradient seems to be associated with the other gradient determined by the summer water table depth and negatively correlated with the organic matter accumulation, soil moisture and altitude. However, this assertion should be treated cautiously because the variable WT usually shows a high spatial and temporal variability (WHEELER & PROCTOR, 2000).

Nevertheless, there is not a complete correspondence between vegetation and edaphic gradients. For example, floristically close associations may be found in habitats with considerable differences in edaphic characteristics. Hence, apart from the soil characteristics there are other factors that determine the vegetation in a place (e.g. topography, land use, history).

The information from the plant assemblages seems to be a better predictor of butterfly composition than edaphic factors (SAWCHIK et al., 2003). Vegetation integrates many ecological factors that may determine the distribution and abundance of butterfly species. Some plants represent essential resources for the survival and reproduction of butterflies. This is notably the case of the host plants, which represent the larval resources. Significant correlations have been detected between butterfly species and their respective host plants (SAWCHIK et al., 2003). Other plants may provide nectar sources for the adults as well as the conditions necessary for sun-basking or mating places. On the other hand, the presence of some plants can also correlate with peculiar ecological situations that are favourable to butterfly species, although this does not imply any causal relationship about their simultaneous presence. In summary, the important associations that exist between butterflies and plant assemblages make the former valuable indicators of habitat types.

Butterfly assemblages and indicator species

The butterfly assemblages are structured among a gradient from lowland eutrophic grasslands to high altitude oligotrophic bogs. Three main butterfly assemblages were identified, which showed characteristic sets of indicator species. Five butterfly species were identified as characteristic of the different assemblages : *B. ino, C. selene, L. helle, L. hippothoe*, and *P. eunomia*. As a result of the variation in their optima, the relative abundances of these five species changed gradually along the main ecocline. Therefore, the composition of the assemblages changes principally according to the dominance structure of these species. The other butterfly species are in general more widespread, generalist or ubiquitous.

One assemblage is characterized by the strong abundance of *C. selene* and, in lesser extent by *L. hippothoe*. This assemblage is typical of oligotrophic sites (bogs and wet rush meadows). A second assemblage, essentially consisting of bistort meadows is characterized by high numbers of *L. helle* and *P. eunomia*. These were both the most diverse assemblages and showed a lesser dominant structure in distribution of species abundances. The third group, typified by meadowsweet grasslands is dominated by *B. ino*. The vegetation of these sites is dominated by tall herbs like *Angelica sylvestris*, *Cirsium palustris*, *Lysimachia vulgaris*, and *Valeriana repens*. These categories seem to be clearly structured along a vegetation gradient, showing various intermediate habitat types. In summary, one extreme of the gradient is represented by peat bogs, followed successively by oligotrophic rush meadows, bistort meadows, meadowsweet grasslands and highly eutrophic wet sites at the other extreme. The latter are represented by species-poor grasslands dominated by the common nettle (*U. dioica*) and with low biological value (i.e., low butterfly species richness and abundance). All along this gradient, the composition of butterfly assemblages' changes gradually from sites dominated by *C. selene* to sites dominates by *B. ino*.

Ecological breadths of the species vary from the more eurytopic ones, typical of high-level structure (e.g., *B. ino*), to stenotopic species characteristic of some well-defined groups (e.g., *C. selene, P. eunomia*). The presence of *B. ino* seems to be assured by the presence of the meadowsweet (*F. ulmaria*), its larva host-plant. This plant is well represented in different types of semi-natural meadows comprising eutrophic grasslands, bistort meadows, and wet peaty meadowsweet grasslands.

The ecological niches of the five indicator species are probably confined to wet grasslands and they are rarely observed elsewhere. On the other hand, field observations reveal that the butterflies identified as detector species (*A. levana* and *A. Crataegi*) also occupy drier grasslands. Detector species are defined by moderate levels of fidelity and specificity. Changes in abundance of these species may provide information on the direction of ecological change (MCGEOCH et al., 2002). In particular *A. levana*, which feeds principally on *U. dioica*, shows a higher tolerance than the other mentioned species (OOSTERMEIJER & VAN SWAAY, 1998). Although its indicator value is limited in this way, it may be an interesting detector species for identifying changes in the level of eutrophication of the habitats. However, detector species are also more prone to important inter-annual or spatial variations in their IndVal values (MCGEOCH et al., 2002). For this reason, future research should consider the validation of the results with independent data sets.

Our results suggest that the set of five species identified as indicators may constitute a useful tool for conservation purposes. Focusing the conservation efforts on the habitat requirements of these species, may be beneficial to protect a significant proportion of wet meadows. These five species are more or less specialized within distinct

sections of the main gradient and are indicators of particular habitats. Therefore, if we preserve and manage refuge sites for these species we are likely to be providing protection for other organisms living in the same biotopes. Concentrating management practices on these five butterfly species will also result in cost-effective administration of time and funding resources.

The five indicator species show features that make them ideal candidates for focal species. They form relatively large colonies in spite of being more or less threatened species (GOFFART et al., 1992). They may be assessed quickly with cheap and standard methods. Moreover, some of these species show narrow tolerances so they may be particularly sensitive to environmental changes (OOSTERMEIJER & VAN SWAAY, 1998). By using a multi-species approach we are covering a long gradient of environmental conditions. The five indicator species encompass al the range of the studied biotopes. All the sites showed the presence of at least one of these species. The simultaneous presence of many of these species may be an indicator habitat heterogeneity.

Indicator and umbrella species are not equivalent concepts, and may be interesting complementary tools for conservation practices (FLEISHMAN et al., 2000). However, some particular species may constitute indicator as well as umbrella species. For example, the 5 species identified as indicators have some characteristics that suggest they may be candidates to conform a suite of umbrella species. They are easily recognizable, show an intermediate degree of rarity, are moderately sensitive to human disturbance, and encompass a large range of habitats (FLEISHMAN et al., 2000; MAES, 2004). However, to be considered as umbrella species, they must show a high pattern of co-occurrence with many other typical species and that was not tested in the present study. *Proclossiana eunomia* and *L. helle* are probably the best candidates because they were associated to the second correspondence axis, which was significantly correlated with species richness and diversity. One particular advantage, in the case of *P. eunomia*, is the considerable knowledge about its life-history and ecology (BAGUETTE & NÈVE, 1994; BAGUETTE et al., 1998; NÈVE et al., 1996; PETIT et al., 2001; SCHTICKZELLE et al., 2002).

To conclude, because of the many advantages described above, we propose that *B. ino, C. selene, L. helle, L. hippothoe* and *P. eunomia* may be used as indicators of habitat types, and as surrogate species for conservation efforts. These species are habitat specialist of small size so they represent interesting tools at small spatial scales as those involved in the present article. The use of species assemblages as indicators may be considerably improved by extending the approach to organisms that are taxonomically and functionally different (MAES, 2004). Future research should be oriented to integrate over larger spatial scales by incorporating knowledge from other taxonomic groups such as birds.

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Seven new species of land planarian from Japan and China (Platyhelminthes, Tricladida, Bipaliidae), with a morphological review of all Japanese bipaliids and a biogeographic overview of Far Eastern species

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ABSTRACT. An account is given of seven new species of land planarian of the family Bipaliidae von Graff, 1896 collected from various localities in Japan and China : three new species of the genus *Bipalium* Stimpson, 1857 and two new species of the genus *Novibipalium* Kawakatsu, Ogren & Froehlich, 1998 from Japan, and two new species of *Bipalium* from China. The bipaliid fauna of Japan and neighbouring countries (South Korea, Taiwan, NE China, and Primorskiy in Russia) is reviewed. The study concludes with a detailed distribution map of this group of animals in the Far East.

KEY WORDS : Platyhelminthes, Bipaliidae, *Bipalium*, *Novibipalium*, *Diversibipalium*, Japan, Korea, Taiwan, China, Primorskiy, taxonomy, morphology, biogeography.

INTRODUCTION

William Stimpson, an American naturalist who visited Japan in 1853 and 1854, was the first scholar who reported on Japanese land planarians, describing four bipaliid species and one geoplanid species (STIMPSON, 1857). His contribution was followed by VON GRAFF'S (1899) Monograph, describing two bipaliid species from Japan (one species concerning a misidentification), and a paper by KABURAKI (1922a) reporting ten bipaliids, two rhynchodemids, and a single geoplanid species, including STIMPSON'S uncertain species. Although the last-mentioned publication contains errors in species identification, it became the fundamental literature on the terrestrial planarians in Japan until recently (cf. KAWAKATSU, 1991a; see also the web article by KAWAKATSU & SASAKI, 2001).

More modern revisions of a few Japanese bipaliid species were made by several workers (MACK-FIRA & KAWAKATSU, 1972; KAWAKATSU, 1991b; KAWAKATSU & KAWAKATSU, 1991) and two new Japanese bipaliid species were reported by KAWAKATSU et al. (1982) and MAKINO & SHIRASAWA (1983).

More recently, the Land Planarian Indices Series has promoted the taxonomic study of Japanese land planarians (see under URL's in References). In addition to the bibliographic clarification offered in these indices of all known Japanese terricolans, they also provided a taxonomic revision of the entire genus *Bipalium* s.l. Stimpson, 1857 and erected the new Oriental genus *Novibipalium* Kawakatsu, Ogren & Froehlich, 1998 (see also KAWAKATSU & OGREN, 1998a, b). In recent issues of the Land Planarian Indices Series the new collective (genus) group *Diversibipalium* Kawakatsu, Ogren, Froehlich & Sasaki, 2002 was created to contain uncertain bipaliid species that cannot be classified in the genera *Bipalium*, *Novibipalium,* and *Humbertium* Ogren & Sluys, 2001 (KAWAKATSU et al., 2002). A distribution map for the Bipaliidae was published by OGREN et al. (1992).

In the present paper we contribute further to the knowledge on the diversity of Japanese and Chinese terricolans by describing five new species of the genus *Bipalium*, two new species of the genus *Novibipalium*, and by providing a morphological, anatomical, and biogeographic review of all bipaliid species from Japan, as well as several species from SE Asia, thus facilitating identification of future samples of these animals.

MATERIAL AND METHODS

Prior to embedding in Paraplast, preserved specimens were first soaked in a mixture with equivalent parts of glycerin and 70% ethanol for about three months (cf. KAWAKATSU et al., 1981). Subsequently, each specimen was divided transversally into 2-4 pieces according to the size of the animals : head-and-prepharyngeal piece (HP, or PRE), pharynx-and-copulatory/genital apparatus piece (labelled as PC, PG, or PHG), and tail piece (T). Relatively short animals were cut into two pieces, viz. HP and PC (also labelled as GT or CT), the latter including the tail. In rather long specimens the PC part was divided into

two sections, one containing the pharynx (P), the other the copulatory/gential apparatus + tail (CT, or GT).

Embedded pieces were serially sectioned at intervals of 7-8 µm. Generally, each of the pieces was sectioned sagittally. A short part taken from the posterior end of the HP piece (i.e. just anterior to the root of the pharynx) was sectioned transversally for examination of the body musculature; this is the prepharyngeal transverse piece (PRE). The serial sections were stained with Delafield's hematoxylin and erythrosin.

The material is deposited in the Zoological Museum of the University of Amsterdam (ZMA) and the National Science Museum in Tokyo (NSMT).

Abbreviations used for expressing the dimensions of the preserved specimens are as follows : TL, total length; HML, distance from the anterior tip to the mouth; HGL, distance from the anterior tip to the genital pore; WM, width over the mouth; TB, thickness of the body at the level just anterior to the pharyngeal region.

Abbreviations used in the figures

au, auricle; bc, bulbar cavity; ca, common genital atrium; cs, creeping sole; csd, common sperm duct; dos, dorsal surface; dst, dorsal stripes; e, eye(s); ed, ejaculatory duct; eg, erythrophilic glands; fa, female genital antrum; fgd, female genital duct; fgp, female genital pore; gl, glands; gp, gonopore; h, head plate; i, intestine; ma, male genital antrum; mgp, male genital pore; od, ovovitelline duct; p, parasite; pb, penis bulb; pg, penis glands; pp, penis papilla; ps, penis sheath; sd, sperm duct; sv, seminal vesicle; te, testis; vs, ventral surface.

A note on anatomical terminology

In previous papers of the senior author, the female copulatory apparatus was described with the terms glandular chamber or glandular duct, following VON GRAFF'S (1899) usage of the German term Drüsengang. In this paper we refer to this particular part of the copulatory apparatus as the female genital duct. VON GRAFF used the term Drüsengang, or glandular duct, for any duct receiving the openings of shell glands. Thus, a Drüsengang could refer to separate oviducts receiving shell glands, a common oviduct receiving the shell glands, or another duct penetrated by gland openings. However, a common oviduct, for example, should not be called a glandular duct simply because it receives the shell glands. Homologous structures should be described with similar terminology. The word Drüsengang or glandular duct refers to several, non-homologous structures.

We presume that in bipaliids the part of the female copulatory apparatus receiving the openings of the oviducts is homologous with the bursal canal of other terricolans. However, in view of the absence of a copulatory bursa, we refrain from using the term bursal canal and instead prefer to use female genital duct. A female genital duct is characterized by a histology and musculature that is different from that of oviducts, common oviduct, and female or common antrum. The term female genital duct has been used in the description of marine triclads that lack a bursal canal (cf. SLUYS, 1989) and was already applied to terricolans by BALL & SLUYS (1990).

SYSTEMATIC ACCOUNT

Suborder TRICLADIDA Lang, 1884

Infraorder TERRICOLA Hallez, 1892

Family BIPALIIDAE von Graff, 1896

Genus ***Bipalium*** Stimpson, 1857

Bipalium tetsuyai sp. nov

Material : Holotype, ZMA V.Pl. 984.1, Mt. Moiwa (alt. approx. 500 m), the western part of Sapporo City, Hokkaidô, 8 August 1966, sagittal sections on 100 slides (HP : 51 slides; PC : 50 slides).

Diagnosis

Bipalium tetsuyai sp. nov. can externally be distinguished from its congeners by its moderate size (60 mm long), lunate head with a blackish margin, uniformly brown dorsal surface with rather narrow blackish mid-dorsal stripe running from the level of the "neck" to the pharynx, and by the absence of stripes on the ventral surface. With respect to anatomical features the new species differs from other species of *Bipalium* in the presence of a large penis bulb, a moderately large conical penis papilla, wide penial lumen with many plicae, spacious and rather muscular male antrum, and a glandular organ that is moderate in size, muscular and houses a moderately glandular female genital duct which is provided with many plicae.

Ecology and distribution

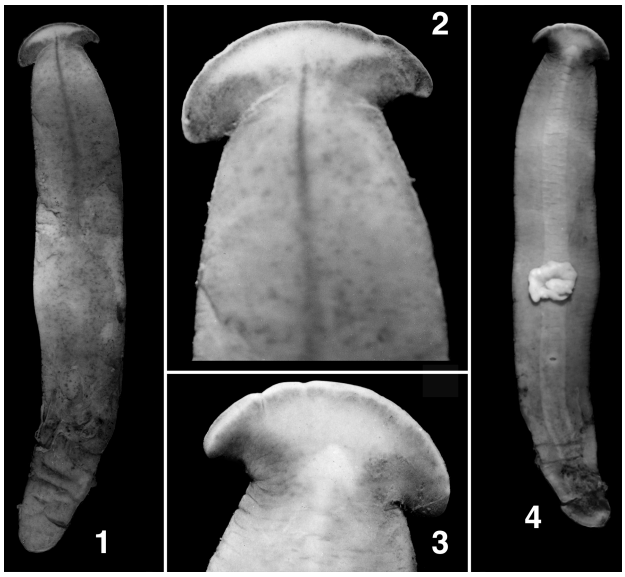
Known only from the type locality.

Etymology

The specific epithet is based on the name of Kawakatsu's son, who was one of the collectors of this new species.

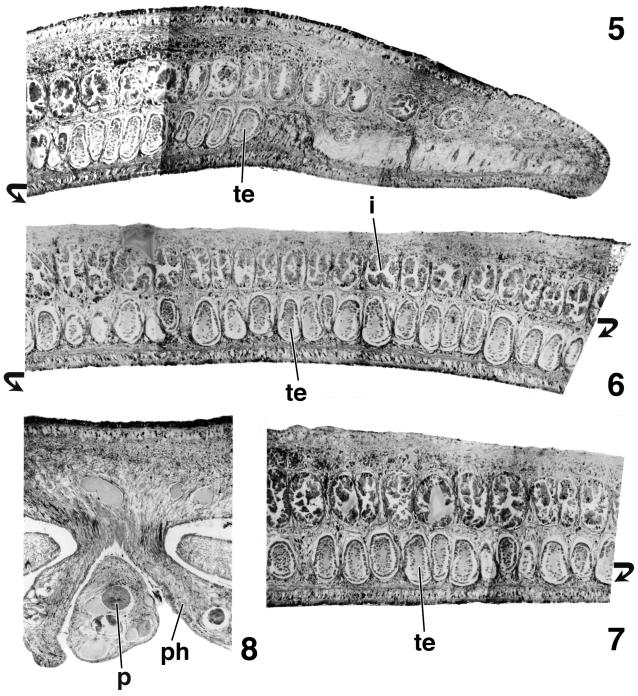
Description

The living, sexually mature specimen in elongated state measured over 60 mm in length. The dimensions of the preserved holotype specimen were as follows : TL : 33 mm, HML : 18 mm, HGL : 23 mm, WM : 2.5 mm. The semi-lunar head has a pair of protruding, moderately recurved auricles (Figs 1-4). Behind the head the body narrows slightly and then gradually widens towards the pharynx and the copulatory apparatus; tail bluntly pointed.



Figs 1-4. – *Bipalium tetsuyai*. ZMA V.Pl. 984.1. 1, dorsal view of preserved specimen; 2, dorsal view of the anterior end; 3, ventral view of the head; 4, ventral view.

The ground colour of the dorsal surface is a uniform brown, except for the whitish head. Auricles and peripheral region of the head are black (Fig. 1). A blackish longitudinal mid-dorsal stripe extends from the middle of the head to the prepharyngeal region. The ventral surface is pale brown, except for the creeping sole and regions over the pharynx and the copulatory apparatus. The margin of the head and basal part of the auricles (i.e. the neck) are greyish black.



Figs 5-8. – *Bipalium tetsuyai*. ZMA V.Pl. 984.1. Sagittal section through front end (5), prepharyngeal region (6, 7), and pharynx (8).

Numerous small eyes are set along the anterior margin of the head in 2-3 or more rows, this "band" of eyes being widest at the regions of the auricles and the "neck".

The ventral testes are rather small and oblong, occupying about one-third of the dorso-ventral diameter in the prepharyngeal part of the body (Figs 5-8). The testes are arranged in one or two longitudinal rows, extending from behind the ovaries to the level of the pharynx or somewhat beyond. Ovaries are located ventrally, near the posterior level of the brain (Fig. 5). Yolk glands are well developed.

The penis bulb is moderately muscular at its ventral side and houses a wide bulbar cavity (or seminal vesicle) with several conspicuous plicae. This bulbar cavity tapers gradually to form an irregularly shaped ejaculatory duct that opens at the tip of the penial papilla. The bulbar cavity and ejaculatory duct are lined with a flat, nucleated and glandular epithelium that is underlain with a thin layer of circular muscle fibres. Posterior and middle sections of the penis lumen receive the numerous openings of erythrophilic glands. The two sperm ducts form well developed spermiducal vesicles, opening separately into the antero-ventral part of the bulbar cavity.

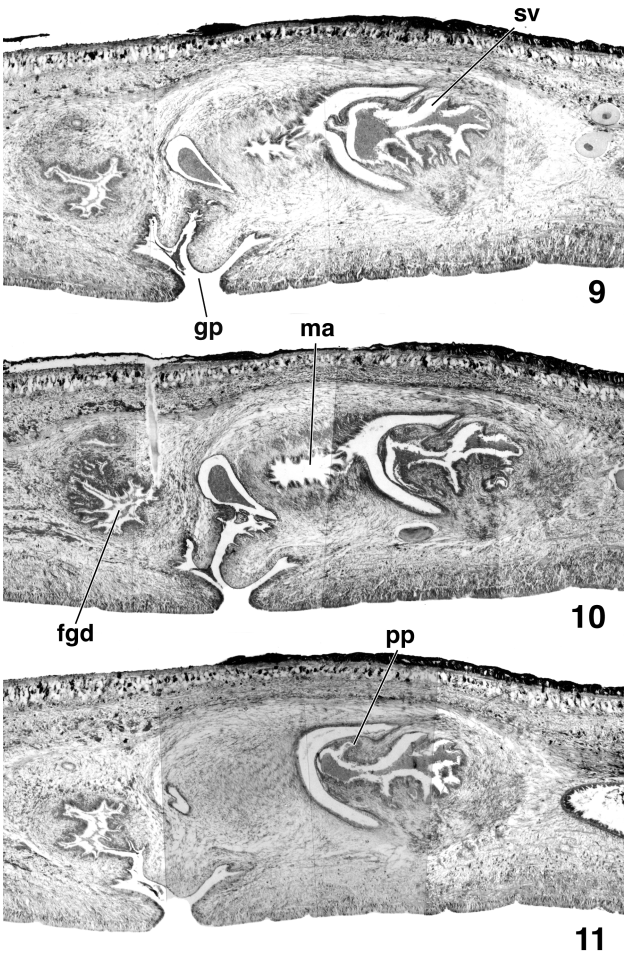


Fig. 9-11. – *Bipalium tetsuyai*. ZMA V.Pl. 948.1 Sagittal sections of the copulatory apparatus; anterior to the right.

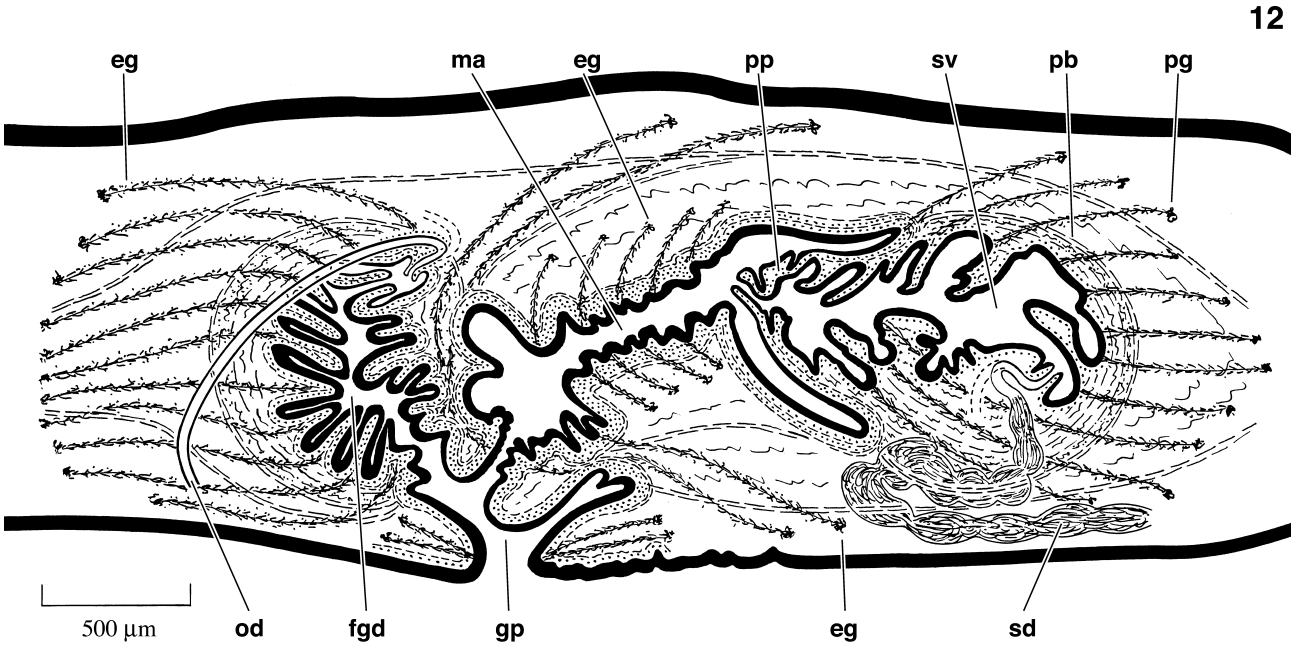


Fig. 12. – *Bipalium tetsuyai*. Holotype. ZMA V.Pl. 984.1. Sagittal reconstruction of the copulatory apparatus; anterior to the right.

The moderately large, conical penis papilla projects into the basal part of the male genital antrum. Judging from the uneven outline of the papilla, it was rather contracted when the animal was fixed (Figs 9-11). The outer wall of the penis papilla is covered with a thin, nucleated epithelium. Near its tip the papilla is provided with a thin, subepithelial layer of circular muscle. In the middle and more basal part of the penis papilla the circular muscle layer is considerably thicker and bounded by a layer of longitudinal muscles.

The irregularly shaped male genital antrum consists of an anterior, proximal cup-shaped cavity, a middle, tubular part provided with plicae, and a posterior, distal section with large folds, communicating with the common genital antrum through a narrow, tubular portion (Fig. 12). The male antrum is lined with a glandular, nucleated epithelium, underlain with a subepithelial circular muscle layer, followed by a thin longitudinal one. On the middle and posterior portions of the antrum the layer of subepithelial circular muscle is thicker than around the anterior part of the antrum; accompanying eosinophilous gland ducts are conspicuous in these areas (Fig. 12).

The rather wide female genital duct is provided with many large plicae and receives the separate openings of the ovovitelline ducts at its anterodorsal section. It is lined with a flat, nucleated and glandular epithelium that is underlain with a thin, subepithelial layer of circular muscle, followed by an equally thin layer of longitudinal muscle fibres. Both layers are thicker at the terminal part of the duct. The female genital duct receives the openings of numerous erythrophilic glands.

The common genital antrum is a shallow, cup-shaped cavity, lined with a flat, glandular epithelium and surrounded by a layer of circular muscle and a layer of longitudinal muscle.

Additionally, the holotype specimen turned out to be infested with a gregarine species of which encysted specimens occur in the parenchyma and the muscular tissue of the pharynx (Fig. 8).

Discussion

Among the 12 known *Bipalium* species from Japan, of which three are new species reported in the present paper, *Bipalium tetsuyai* stands apart from the other species in both external features and anatomy of the genital apparatus (see Diagnosis and Table 1). A lunate head with moderate auricles and a blackish margin (as observed in the living, holotype specimen) represent unique features of this species.

A single, longitudinal blackish mid-dorsal stripe is found also in *B. fuscolineatum*, *B. hilgendorfi*, *B. kisoense*, *B. monolineatum*, *B. ochroleucum*, and *B. tetsuyai;* all these species are devoid of stripes on the ventral surface. However, in *B. tetsuyai* this mid-dorsal stripe runs only between the "neck" and the pharynx, in contrast to the other species, in which this stripe reaches the posterior end of the body.

The anatomy of both female and male genital apparatus of *B. tetsuyai* is similar to that of *B. hilgendorfi* (cf. KABURAKI, 1922a; KAWAKATSU & KAWAKATSU, 1991, Fig. 4A, B; see also Table 1). However, the penis lumen of *B. tetsuyai* is wide and provided with many large plicae, in contrast to the more regularly shaped lumen in *B. hilgendorfi*. With respect to external features it should be noted that in *B. hilgendorfi* the head is rotundate and provided with short auricles, contrasting with the semilunate head of *B. tetsuyai*.

B. tetsuyai can be distinguished from the Korean species *B. koreense* and the Chinese species *B. kaburakii* and *B. katoi* by the fact that these species have a single middorsal stripe, as well as two lateral and two marginal longitudinal stripes (see Table 4).

Bipalium glandiantrum sp. nov.

Material : Holotype, ZMA V.Pl. 985.1, Sanjô City, Nîgata Pref., Chûbu Region, Honshû, 3-6 June 1987, sagittal sections on 28 slides (PRE), and sagittal sections on 31 slides (GT).

Paratypes : ZMA V.Pl. 985.2, ibid., sagittal sections on 14 slides (PRE); V. Pl. 985.2, ibid., transverse sections on 17 slides (PT); V.Pl. 985.2, sagittal sections on 35 slides (G).

NSMT 5417, ibid., sagittal sections on 25 slides (PRE); NSMT 5417, ibid., sagittal sections on 30 slides (CT).

Other material examined : ZMA V.Pl. 986.1, Sanjô City, Nîgata Pref., Chûbu Region, Honshû, 21 July 1987, sagittal sections on 19 slides (PRE); V.Pl. 986.1, ibid., sagittal sections on 18 slides (CT); V.Pl. 986.2, ibid., whole mount on 1 slide.

Diagnosis

Bipalium glandiantrum sp. nov. can be distinguished from its congeners by its small size (30 – 50 mm long), semilunate head with recurved auricles, uniform ochre or yellowish brown dorsal body surface with relatively broad, blackish middorsal stripe and a pair of lateral stripes, dark colouration on submarginal ventral part of the head and on both sides of the neck. The species can be distinguished by a moderately sized penis bulb, elongated conical penial papilla, long and tubular penis lumen, and moderately sized male antrum, spherical female organ with a spacious female genital duct provided with several plicae, common genital antrum anteriorly provided with an adenodactyl-like structure. Furthermore, *B. glandiantrum* sp. nov. has a diploid complement with 2n = 10 chromosomes.

Ecology and distribution

Known only from the type locality, where it was collected from the garden of Mr. Kozakai's residence.

Etymology

The specific epithet is derived from the Latin prefix *glandi*, meaning "glandular" and the noun *antrum*, "cave". It alludes to the glandular nature of the anterior part of the common genital antrum.

Description

A rather small species. According to Mr. Murayama, one of the collectors, the largest sexual specimen in elongated state was approximately 50 mm in length with a width of about 5mm. The body dimensions of four preserved specimens are as follows : TL : 29-38 mm, HML : 14-18 mm, HGL : 17-23 mm, WM : 3.5-4 mm.

In the living animal the semi-lunar head shows a pair of protruding and recurved auricles. In preserved condition the depressed head is rotundate with short and rounded auricles. The body narrows slightly at the region of the "neck", and subsequently broadens again towards the pharynx and the copulatory apparatus; the tail is bluntly pointed. In preserved specimens the body has a serrate outline (Figs 13, 14).

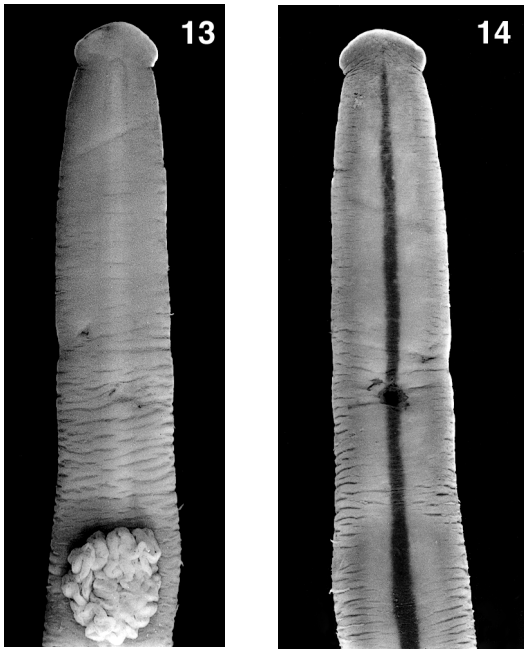


Fig. 13-14. – *Bipalium glandiantrum*. ZMA V.Pl. 985. Ventral (13) and dorsal (14) views of preserved specimen.

The dorsal surface has a uniform ochre or yellowish brown ground colour; the head is darkish, except for its margin. The dorsal surface is provided with three longitudinal stripes. There is a black and relatively broad middorsal stripe, extending over the entire body, except for the anterior part of the head plate; the stripe becomes slightly broader over the regions of the pharynx and the copulatory apparatus. There are two dark coloured, relatively broad lateral stripes, one on either side of the body, beginning at the "neck" and extending to almost the posterior end of the body.

The ventral body surface has a uniformly pale colouration, except for the creeping sole, the submarginal parts of the head and both sides of the "neck" that show a dark colouration; furthermore, a pair of brownish, relatively wide lateral longitudinal stripes is conspicuous in living specimens.

A few rows of numerous small eyes are irregularly distributed along the margin of the head.

The testis follicles are situated ventrally in the prepharyngeal part of the body, but are only well developed (albeit not fully mature) in specimen V.PI.986.1 (Fig. 15).

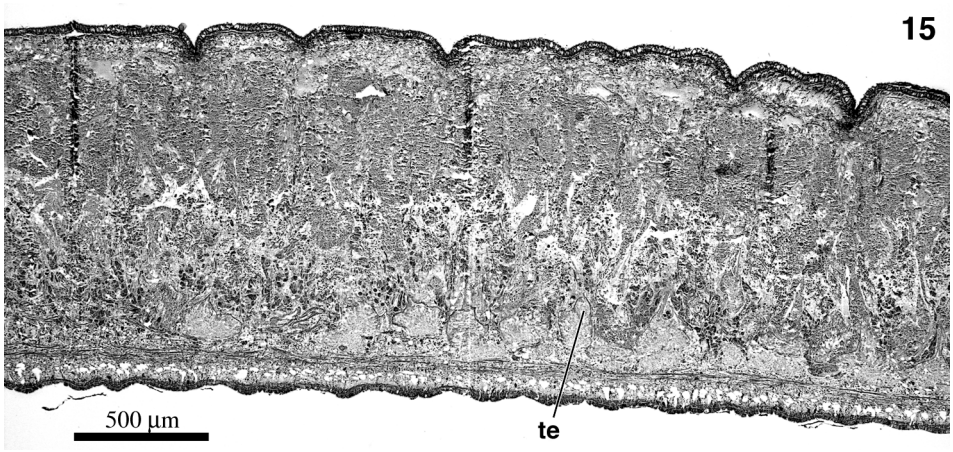


Fig. 15. – *Bipalium glandiantrum*. ZMA V.Pl. 986.1. Sagittal section of the prepharyngeal part; anterior to the right; notice testes.

The penis consists of a moderately large spherical bulb and an elongated, conical papilla. The bulb is moderately muscular and houses a narrow, tubular cavity. The latter receives the separate openings of the sperm ducts at its anterior part, while the posterior section of the cavity communicates with the club-shaped ejaculatory duct, which opens at the tip of the penis papilla (Figs 16, 17). The bulbar cavity and the ejaculatory duct are lined with a nucleated, glandular epithelium that is underlain with a subepithelial layer of circular muscle, followed by a layer of longitudinal muscle fibres. Erythrophilic penis glands open into the bulbar cavity and into the basal portion of the ejaculatory duct. The penis papilla is covered with a thin, nucleated epithelium, underlain with a thin, subepithelial layer of circular muscle, being rather thick at the base of the papilla, followed by an equally thin layer of longitudinal muscles**.**

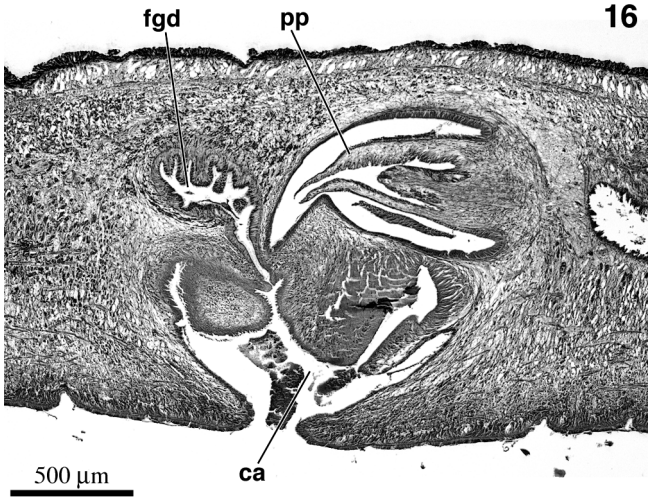


Fig. 16. – *Bipalium glandiantrum*. NSMT 5417. Sagittal section of the copulatory apparatus; anterior to the right.

The male genital antrum, with the shape of a champagne glass, communicates with the dorsal section of the posterior part of the common atrium (Fig. 17). The male antrum is lined with a thin, nucleated epithelium, underlain with a thin, subepithelial layer of circular muscle, followed by a thin layer of longitudinal muscles.

The female genital duct consists of two parts : a wide posterior part provided with several well-developed plicae, and an anterior section that ventrally opens into the posterodorsal part of the common antrum (Fig. 17). Two ovovitelline ducts open separately into the posterior part of the female genital duct. The small ovaries are situated immediately above and slightly embedded in the thickened sections of the ventral nerve cords that form the brain. Yolk glands are well developed and lie dispersed in the parenchyma.

The female genital duct is lined with a rather flat, nucleated epithelium and is surrounded by a subepithelial layer of circular muscle and a layer of longitudinal muscle fibres. Over its entire length the duct receives the numerous openings of erythrophilic glands.

The common genital atrium is formed by a shallow cup-shaped cavity that opens into the gonopore.

Ventrally to the male antrum, a hump-shaped adenodactyl-like organ is differentiated, consisting of loosely arranged muscles and a rather wide lumen. This lumen is lined with a flat, infranucleated epithelium and is surrounded by a relatively thick subepithelial layer of circular muscle, followed by a thin layer of longitudinal fibres. The lumen receives the numerous openings of erythrophilic and cyanophilic glands; it opens into the anterior part of the common antrum.

The posterior section of the common antrum adjacent to the adenodactyl-like organ is provided with a thick layer of circular muscle fibres, followed by a thinner layer of longitudinal fibres. The entire common antrum is lined with a nucleated epithelium.

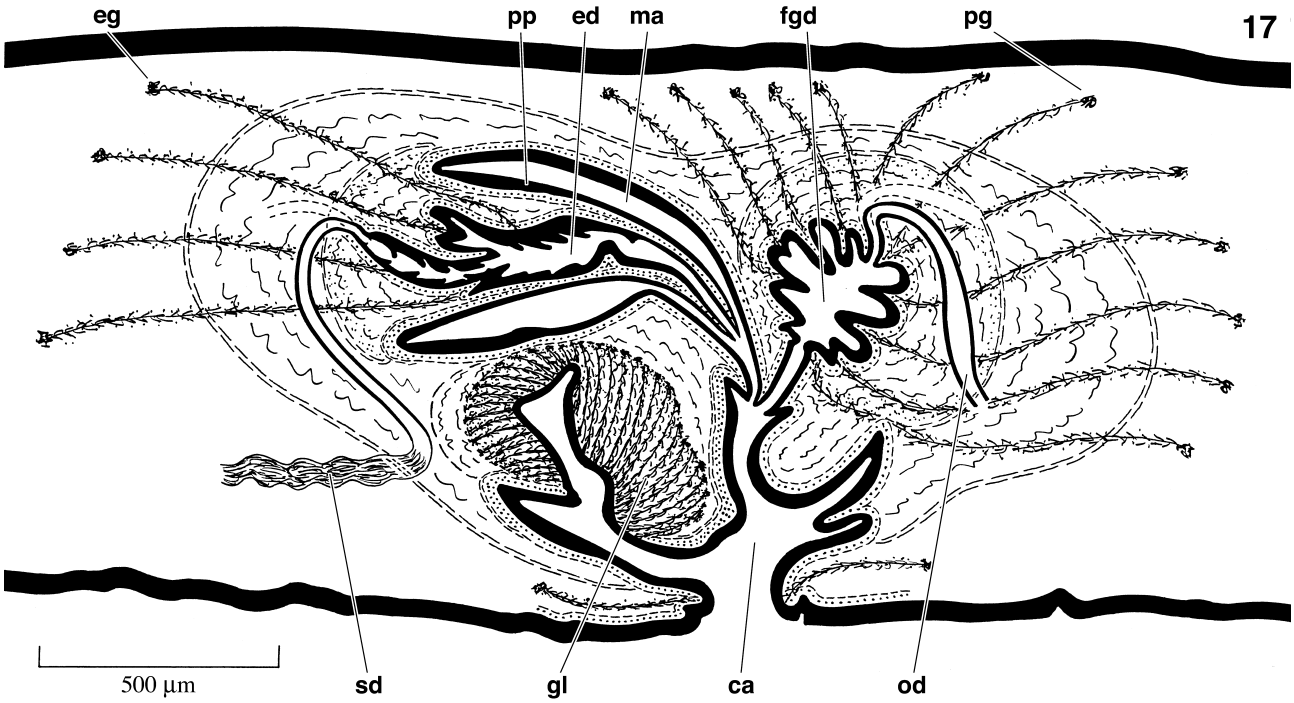


Fig. 17. – *Bipalium glandiantrum*. Holotype. ZMA V.Pl. 985.1. Sagittal reconstruction of the copulatory apparatus; anterior to the left.

Discussion

This species can be distinguished from the other eleven Japanese species of *Bipalium* by its rather small body size, semilunate head with moderately recurved auricles, yellowish brown dorsal surface with three longitudinal stripes, and a pair of brownish longitidinal lateral stripes on the ventral surface (Table 1); these external features are unique for *B. glandiantrum*.

With respect to its external features, *B. glandiantrum* resembles somewhat *Novibipalium trifuscostriatum* (Kaburaki, 1922) (cf. KAWAKATSU, 1991b, figs 1A-C). However, the latter is a dark brown species with three very conspicuous, blackish longitudinal stripes on the dorsal surface, while the ventral surface is without stripes (see Tables 1 and 2). On the basis of their genital anatomy, these two species belong to different genera.

Characteristic for *B. glandiantrum* is its common genital antrum provided with an adenodactyl-like structure not found in any other Japanese species of *Bipalium* (cf. Table 1). In *B. nobile* and *B. kewense* there is a small glandular structure situated in the roof of the common genital antrum but morphologically and histologically these glandular organs are unlike the adenodactyl-like structure in *B. glandiantrum* (cf. KAWAKATSU, 1985, Fig. 4; KAWAKATSU et al., 1982, figs 6A, C).

Bipalium muninense sp. nov.

Material : Holotype, ZMA V.Pl. 987.1, October-November 1987, Ômura, Chichi-jima Island, the Ogasawara Islands, Japan, sagittal sections on 16 slides (PRE); V.Pl. 987.1, ibid., sagittal sections on 43 slides (PHG).

Paratypes : ZMA V.Pl. 987.2, ibid., sagittal sections on 26 slides (PG); V.Pl. 987.2, ibid., transverse sections on 17 slides (PRE); NSMT 5418, ibid., sagittal sections on 22 slides (PG); ZMA V.Pl. 987.3, ibid., sagittal sections on 18 slides (PG); V.Pl. 987.4, whole mount on 1 slide.

Diagnosis

With respect to external features, *Bipalium muninense* sp. nov. can be distinguished from its congeners by its small size (less than 50 mm in length), lunate head with non-recurved auricles, slightly reddish dark brown dorsal surface, yellowish brown head plate, and five dark brown stripes arranged as follows : slender mid-dorsal stripe that extends on the head as an oblanceolate spot; a broad lateral stripe on each side of the median one and beginning at the "neck"; a pair of broad submarginal stripes. Ventral surface devoid of stripes. Anatomically the species is characterized by ellipsoidal, moderately sized penis bulb, short conical penis papilla, spacious penial lumen, well muscularized male antrum at its posterior section, and an oblanceolate female organ provided with a spacious female genital duct.

Ecology and distribution

Known only from the type locality, a subtropical forest at Ômura on Chichi-jima Island (cf. KAWAKATSU et al., 1999, fig. 1).

Etymology

The specific epithet is derived from Munin-jima Island(s), the oldest name for the Ogasawara Islands. "Munin" (or "Mujin") in the Japanese language meaning "without residents", the English name "Bonin Islands" being derived from the Japanese name for these islands.

Description

A rather small species. According to the information provided by the collectors, the largest living, sexually mature specimen reached a length of 50 mm in elongated state. The dimensions taken from three preserved specimens are as follows : TL : 24-33 mm; HML : 11-13 mm; HGL : 16-22 mm; WM : 3-4 mm.

In the living animal the lunate head has a pair of moderately developed, non-recurved auricles (Fig. 18). In preserved conditon the head has a more reniform shape (Figs 19, 20). The body narrows abruptly at the level of the "neck", and subsequently widens gradually towards the region of the pharynx and the copulatory apparatus; the tail is bluntly pointed.

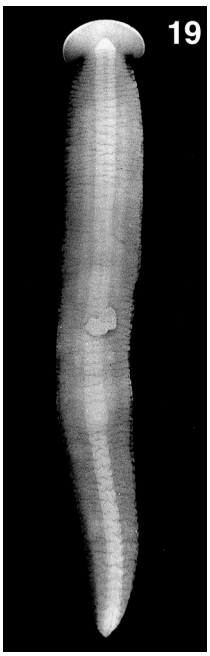
The ground colour of the dorsal surface is rather dark brown, with reddish tint, except for the head plate which shows a yellowish brown colouration; the central and posterior parts of the head plate have a brownish tint. Five dark brown conspicuous dorsal stripes extend to almost the posterior end of the body. A mid-dorsal stripe starts as an oblanceolate spot on the head and extends as a narrow line to the posterior end of the body. This median stripe is flanked by lateral stripes that begin at the level of the "neck" and stop short of the hind end of the body. Along the body margins runs a rather broad submarginal stripe from the "neck" up to the posterior end. The ventral side shows a much paler ground colour, with only the creeping sole being bordered by an indistinct dark pigmentation and the lateral body margins being provided with an equally indistinct pigment pattern.

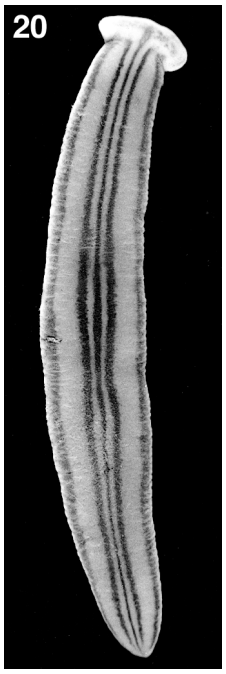
Three or more rows with numerous eyes situated along the periphery of the head, particularly concentrated in the "neck" region (also on the ventral side). The eyes extend posteriorly along the sides of the body but there they are fewer in number, more aligned in a single row, and spaced further apart.

Numerous, rounded testes are situated ventrally, extending from behind the ovaries to the level of the pharynx (Fig. 21).

The penis is provided with a moderately large, ellipsoidal bulb and a conical papilla. The bulb is moderately muscular and houses a very large, irregularly-shaped, obpyriform cavity that anteroventrally receives the separate openings of the two sperm ducts. The bulbar cavity extends into the penis papilla, gradually tapering to give rise to a wide, tubular ejaculatory duct that opens at the tip of the papilla. The entire penis lumen is lined with a tall, nucleated epithelium that is underlain with a subepithelial layer of circular muscle fibres followed by a layer of longitudinal muscle (partly intermingled circular and longitudinal muscle fibres at the basal part of the bulbar cavity).

The penis papilla is covered with a thin, nucleated epithelium and is provided with a thin, subepithelial layer of circular muscle, followed by an equally thin layer of longitudinal muscle fibres.





Figs 18-20. – *Bipalium muninense*. 18, live specimen. Ventral (19) and dorsal (20) views of preserved specimen.

The male genital antrum consists of an anterior, cup-shaped cavity housing the penis papilla and a posterior, lenticular part that opens into the male genital pore (Fig. 22). The anteror part of the male antrum is lined with a thin, glandular and nucleated epithelium, underlain with a thin, subepthelial layer of circular muscle and an equally thin layer of longitudinal muscle fibres. Both the glandu-

lar epithelium and the muscular coat become thicker on the posterior section of the male antrum. The musculature around this posterior part consists of a thick, subepithelial layer of circular muscle, a thick layer of intermingled muscle, followed by an outer layer of longitudinal muscle fibres. Erythrophilic gland ducts open into the bulbar cavity, into the posterior part of the male antrum, and into the anterior part of the male antrum.

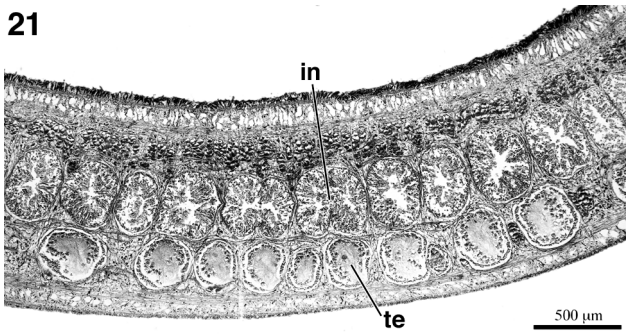


Fig. 21. – *Bipalium muninense*. ZMA V.Pl. 987.1. Sagittal section of the prepharyngeal region; anterior to the left; notice testes.

The small ovaries are situated immediately above and slightly embedded in the thickened sections of the ventral nerve cords that form the posterior part of the brain. Yolk glands are well developed and lie dispersed in the parenchyma.

The female genital duct consists of two parts, viz. a narrow posterior cavity with several plicae and an anterior part that opens into the posteroventral section of the female genital pore. The posterior part of the female genital duct receives the separate openings of the ovovitelline ducts, the latter approaching the duct from posterolateral direction. Many erythrophilic and a few cyanophilic gland ducts penetrate the wall of the female genital duct, excepting its terminal, ventral part. The entire female duct is lined with a relatively tall, glandular and nucleated epithelium; it is surrounded by a thin subepithelial layer of circular muscle, followed by an equally thin layer of longitudinal muscle fibres.

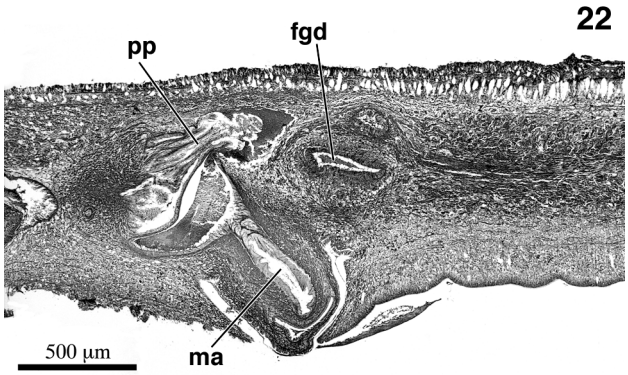


Fig. 22. – *Bipalium muninense*. ZMA V.Pl. 987.3. Sagittal section of the copulatory apparatus; anterior to the left.

In the holotype specimen (Fig. 23), a common genital antrum is hardly developed, except for its anterior portion, due to the elongation of the posterior part of the male antrum and the tubular portion of the female genital duct. The common antrum is lined with a nucleate epithelium that is underlain with a subepithelial layer of circular muscle and a layer of longitudinal muscles.

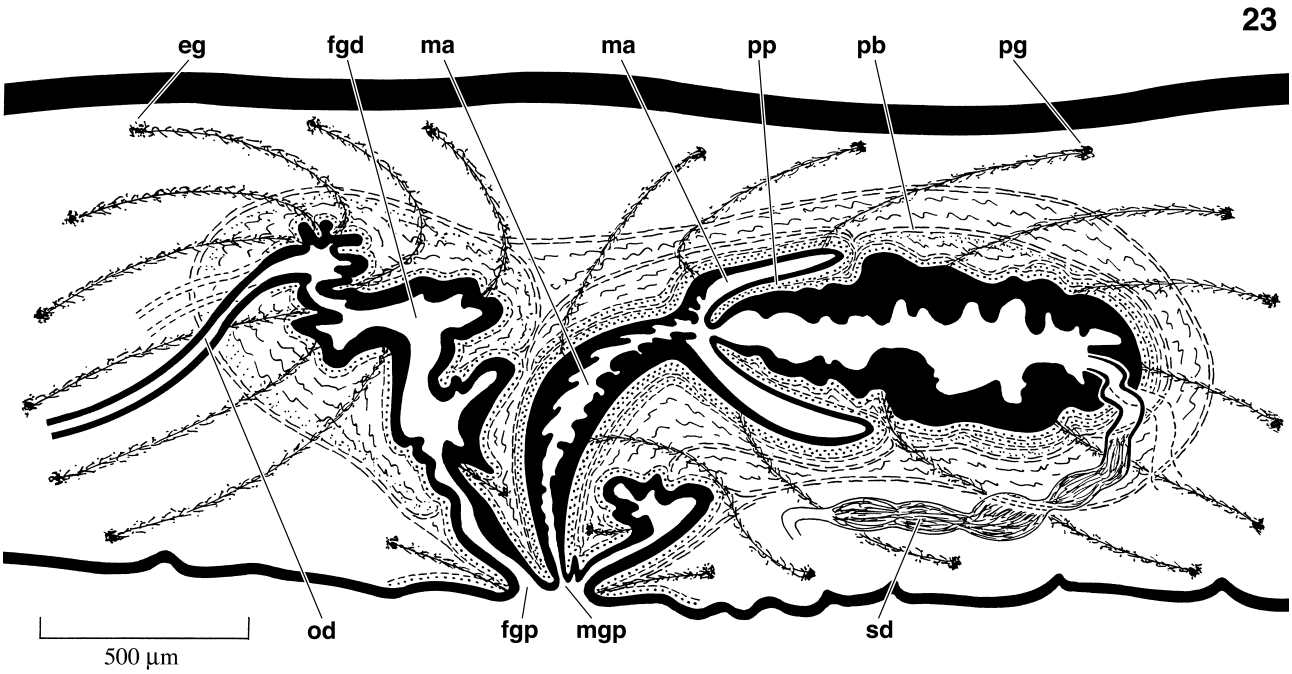


Fig. 23. – *Bipalium muninense*. Holotype. ZMA V.Pl. 987.1. Sagittal reconstruction of the copulatory apparatus; anterior to the right.

Karyology

Chromosome complement : 2x = 10, with a karyotype of 2m + 2sm + 2sm + 2sm + 2m (cf. KAWAKATSU et al., 1990, figs 15D, 16 : *Bipalium* sp. 3; OKI et al., 1991, figs 2C, 3 : *Bipalium* sp. 3).

Discussion

This species can easily be distinguished from other Japanese species of *Bipalium* by its rather small size, lunate head with non-recurved auricles, slightly reddish brown dorsal surface with five longitudinal dark brown stripes, and the absence of stripes on the ventral body surface (Fig. 20; see also Table 1).

Although *B. kewense* also shows five longitudinal dorsal stripes, their pattern is completely different from that in *B. muninense* (cf. KAWAKATSU et al., 1982, figs 3A-C, E, H-I). Furthermore, *B. kewense* possesses ventrally a pair of lateral stripes (cf. KAWAKATSU et al., 1982, figs 3D, F, J). Both the dorsal and ventral patterns of longitudinal stripes in *B. nobile* are quite different from the situation in *B. muninense* (cf. KAWAKATSU et al., 1982, figs 1A-F, 2; see also web articles by SASAKI et al., 2001; YAMAMOTO et al., 2003). Additionally, *B. kewense* and *B. nobile* are very long or giant species, respectively.

The dorsal pattern of *Diversibipalium multilineatum* (Makino & Shirasawa, 1983) resembles that of *B. muninense*, especially in the thickened anterior section of the mid-dorsal stripe on the head plate. However, *D. multilineatum* has three longitudinal stripes on the ventral surface, contrasting with the non-striped condition in *B. muninense*. Furthermore, *D. mulitlineatum* is a long species.

The Chinese species *B. katoi* also has five dorsal stripes but their pattern is quite different from that in *B. muninense* (cf. KATÔ, 1950, Fig. 4; see also Fig. 26 and Table 4).

The anatomy of the reproductive apparatus of *B. muninense* differs from that in other species, notably in the presence of the well-muscularized anterior section of the male genital antrum.

Bipalium kaburakii sp. nov.

Diagnosis

The dorsal surface is dark brown, with five black stripes, one median and two lateral, which extend almost throughout the whole length of the body. The copulatory apparatus shows the following characteristics : an ovoid penis bulb and a conical papilla; a tubular penis lumen with many plicae; a moderately large female genital duct, the posterior part of the latter receiving the openings of the ovovitelline ducts; ovovitelline ducts approaching the female duct from ventro-lateral direction; common genital antrum shallow and cup-shaped.

Etymology

The new name refers to the late Dr. Tokio Kaburaki, who first reported on these Chinese specimens from Soochow.

Description and comparative discussion

KABURAKI (1922b) reported "*Bipalium cantori* Wright, 1860" from Soochow (=Suzhou; 31º21'N 120º40'E), near Shanghai, Chiangsu Province, SE China. In point of fact, this locality is approximately 50 km NW of WRIGHT'S (1860) locality of "*Dunlopea Cantoria*"(i.e. Ningpo, 29º54'N 121º33'E), which is now classified as *Diversibipalium cantori* Wright, 1860 (see below).

KABURAKI (1922b) described his worms as follows : "In shape the worm conforms to the typical *Bipalium*-outline, with the head, which is semi-lunar, rather less than the breadth of the trunk, and marked off from it by a constriction. One of the two specimens measures about 165 mm long by 5 mm broad. The ground colour of the dorsal surface is dark brown, with five black stripes, one median and two lateral, which extend almost throughout the whole length of the body. The median stripe is very fine and anteriorly merges into the ground colour of the head in association with the fine inner pair. The outer pair at the margin of the body anteriorly terminates at the base of the cephalic lappets and is very faint in the preserved specimens. The ventral surface is of a much lighter colour than the dorsal."

KABURAKI (1922b) did not provide any illustrations of the external features of his Chinese specimens. However, in his description of the habitus of the animal he did not mention the most characteristic features of *D. cantori*, viz. the presence of a dark brown margin on the head plate and the two dark lateral stripes that expand on the head to form beak-shaped spots (see above). In the experience of the senior author, external morphological features correlate with anatomical differences and generally occur only in restricted geographic ranges. As a consequence, stripes and pigmentation patterns on the dorsal surface represent valuable taxonomic characters for species identification.

In conclusion, KABURAKI'S (1922b) "*Bipalium cantori* from Soochow" is here considered to be a misidentification of a new local species. Its copulatory apparatus is characterized by the following features : a moderately large, ovoid penis bulb and a moderately sized conical papilla; a tubular penis lumen with many plicae; a moderately large female genital duct, the posterior part of the latter receiving the openings of the ovovitelline ducts; ovovitelline ducts approaching the female duct from ventro-lateral direction; common genital antrum shallow and cup-shaped (Fig. 24).

The samples of this Chinese animal studied by KABURAKI (1922b) were lost (cf. KAWAKATSU & SASAKI, 2004).

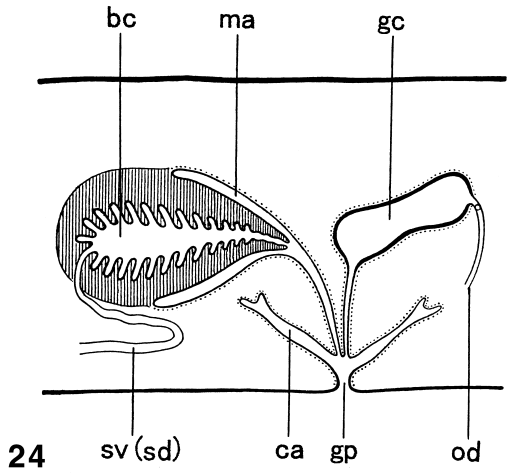


Fig. 24. – *Bipalium kaburakii*. Sagittal view of the copulatory apparatus (after KABURAKI, 1922b); anterior to the right.

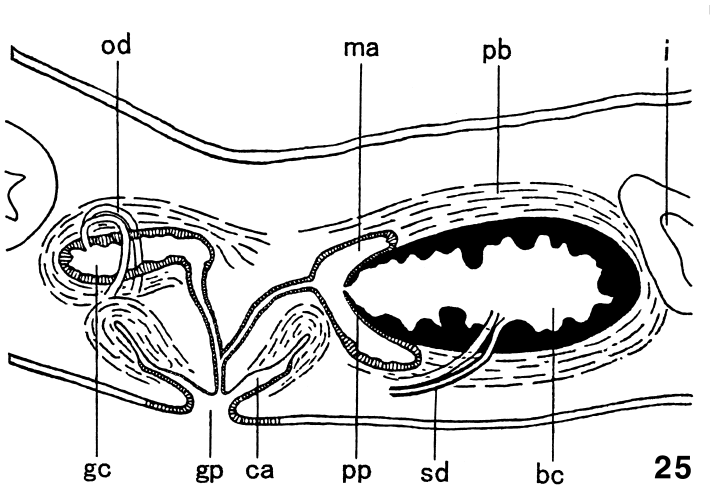


Fig. 25. – *Bipalium katoi*. Sagittal view of the copulatory apparatus (after KATÔ, 1950); anterior to the left.

Bipalium katoi sp. nov.

Diagnosis

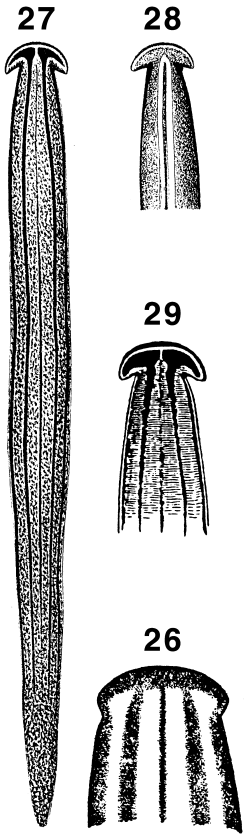
The dorsal surface shows five black, longitudinal stripes : a rather thin middorsal stripe, two broad lateral ones, and two broad marginal stripes; all stripes merge with a wide black band running along the entire margin of the head. The copulatory apparatus is characterized by the following features : short, conical penial papilla; very spacious penis lumen receiving the sperm ducts in its middle section; ovovitelline ducts opening dorso-laterally into the posterior portion of the female genital duct; female genital duct and the posterior, tubular part of the male antrum fuse to form a short common duct that opens at the midventral section of a muscular gential pad.

Etymology

The new name refers to the late Dr. Kojirô Katô, who was the first to describe the animals from Shanxi Province.

Description and comparative discussion

KATÔ (1950) reported a single, sexually mature specimen of "*Bipalium cantori* (Wright, 1860)" from a locality between Huang-shuighen and Huang-lingkuan, Shanxi Province (i.e. between 34º30' – 40º30'N, 109º30' – 113º30'E). Unfortunately, the material is no longer available for study) since Dr. Kato's histological slides were lost during the Second World War (cf. KAWAKATSU & SASAKI, 2004). In preserved condition the animal was 70 mm long and 4 mm wide, with a rounded head. The colour of the body was a blackish brown, but after the animal had been exposed to a clearing agent, five black, longitudinal stripes were observed : a rather thin middorsal stripe, two broad lateral ones, and two broad marginal stripes. All stripes merge with a wide black band running along the entire margin of the head (Fig. 26).



Figs 26-29. – *Bipalium katoi* and *Diversibipalium cantori. B. katoi*. 26 : dorsal view of the head (after Katô, 1950); *D. cantori.* 27 : dorsal view; 28 : ventral view of the head and anterior part of the body (after VON GRAFF, 1899); 29 : dorsal view of the head and the anterior part (after WRIGHT, 1860).

The male copulatory apparatus consists of a spheroidal penis bulb and a short, conical papilla. Both papilla and bulb are provided with a very spacious, plicate lumen. Sperm ducts approach the lumen from ventro-posterior direction and open into the middle section of the penis lumen.

The female genital duct receives the separate openings of the ovovitelline ducts at its dorsolateral section. Female genital duct and male antrum fuse to form a short common duct that opens at the midventral section of a muscular gential pad, the latter projecting into the common antrum. This common antrum is provided with a thick coat of muscle (Fig. 25).

KATÔ (1950) noted that there are several morphological and anatomical differences between his animal from Shanxi Province and KABURAKI'S (1922b) specimens from Chiangsu Province, but he attributed these differences to geographical variation within a single species (the two sampling localities are approximately 1000 km apart).

However, the external morphology of the head and the anterior part of the body in KATÔ'S specimen is quite different from that of *Diversibipalium cantori* and *Bipalium kaburakii* sp. nov. Although the anatomy of the copulatory apparatus of Katô's animal resembles that of *B. kaburakii* sp. nov., the former differs by exhibiting the following features : short, conical penial papilla; very spacious penis lumen receiving the sperm ducts in its middle section; sperm ducts approaching the penis lumen from ventro-lateral direction; ovovitelline ducts opening dorsolaterally into the posterior portion of the female genital duct; female genital duct and the posterior, tubular part of the male antrum fuse to form a short common duct that opens at the midventral section of a muscular gential pad, the latter projecting into the common antrum.

In view of the considerations presented above we conclude that Katô's "*Bipalium cantori*" from Shanxi Province actually represents a new species, for which we here coin the name *Bipalium katoi* sp. nov.

Genus ***Novibipalium***
Kawakatsu, Ogren & Froehlich, 1998

Novibipalium miyukiae sp. nov.

Material : Holotype, ZMA V.Pl. 988.1, 28 October 1983, Hakodate City, Hokkaidô, sagittal sections on 18 slides (H); V.Pl. 988.1, ibid., sagittal sections on 16 slides (PC).

Diagnosis

On external features, *Novibipalium miyukiae* sp. nov. can be distinguished from other species of *Novibipalium* by its small size (preserved, 26 mm), rotundate head, and uniformly dark to blackish brown dorsal surface being devoid of stripes. Anatomically, the species is characterized by the following features : large, spheroidal penis bulb; penis papilla with turbinate base and long, pointed tip; penis lumen consisting of a T-shaped seminal vesicle, a middle section constituting a spacious bulbar cavity with many plicae, and a proximal section forming a tubular ejaculatory duct. The middle and posterior sections of the male antrum form a well-developed penis sheath provided with a long, wide and bellows-shaped copulatory canal that is surrounded by a thick muscle coat. Relatively large, ellipsoidal female organ housing a spacious, irregular female genital duct that opens through a narrow, tubular part into the common antrum.

Ecology and distribution

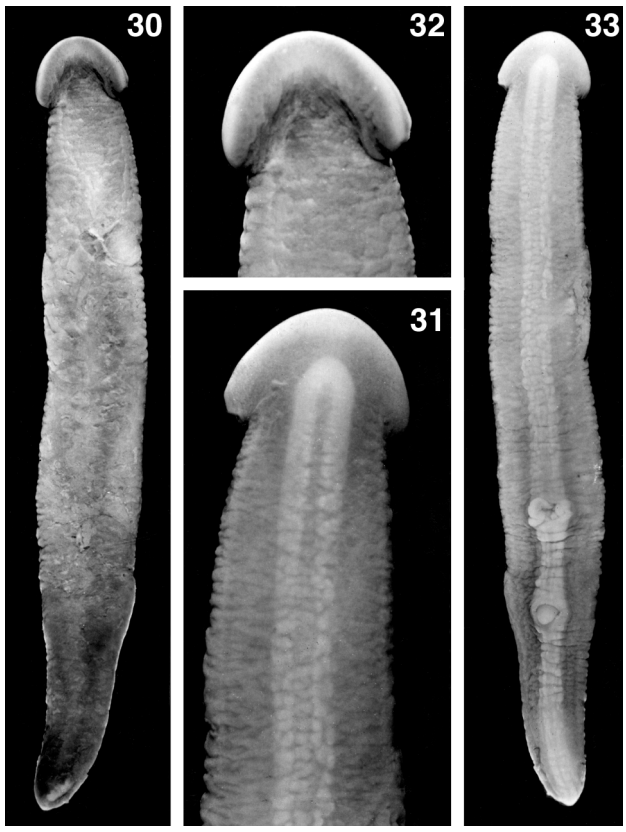
Known only from the type locality, where it was collected from the garden of Dr. Munakata's residence, Kajichô, Hakodate.

Etymology

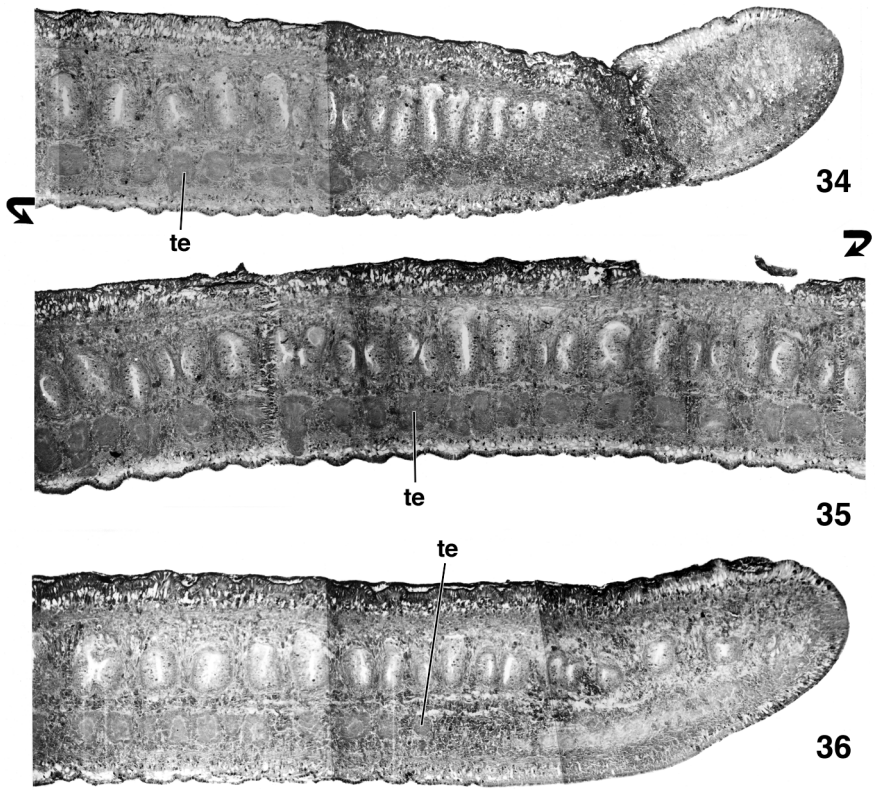
The specific epithet is based on the name of Kawakatsu's daughter, whose technical assistance throughout the years has been invaluable for the turbellarian studies of the senior author.

Description

A small and rather slender species. The dimensions of the preserved holotype specimen were as follows : TL : 26 mm; HML : 16 mm; HGL : 20 mm : WM : 3 mm. The contracted head was acorn-shaped with short, bluntly pointed auricles. Behind the head the body narrows slightly to form a "neck", after which it gradually widens again towards the regions of the pharynx and the copulatory apparatus; tail bluntly pointed. Denticulate lateral margins are conspicuous in the preserved specimen (Figs 30-33).



Figs 30-33. – *Novibipalium miyukiae*. ZMA V.Pl. 988.1. 30, dorsal view of preserved specimen; 31, ventral view of the anterior end; 32, dorsal view of the head; 33, ventral view.



Figs 34-36. *Novibipalium miyukiae*. ZMA V.Pl. 988.1. Sagittal sections of the prepharyngeal part of the animal.

The head plate is dark reddish brown and the dorsal body surface is uniform dark to blackish brown, without stripes. The ventral surface is uniformly greyish brown, except for the wide creeping sole. Numerous small eyes are present along the margin of the head plate, arranged in several rows.

The musculature in the dorsal part of the body is made up of a subepithelial muscle system consisting of a thin layer of circular muscle directly underneath the basement membrane, followed by a thin layer of longitudinal muscle fibres. A submuscular nerve net and a relatively thick layer of longitudinal muscle fibres are located below the subepithelial muscle layers. The body musculature on the ventral surface consists of a thin subepithelial layer of circular muscle, an equally thin layer of longitudinal fibres, a submuscular nerve net, and a thick layer of intermingled circular and longitudinal muscle fibres.

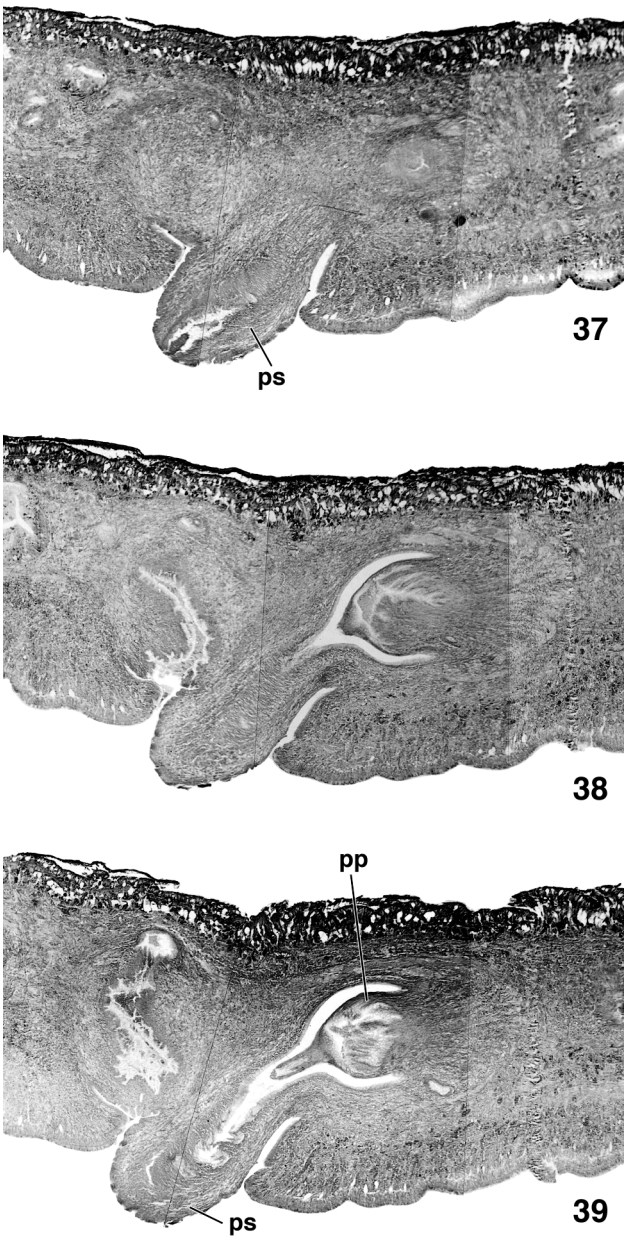
The ventral, prepharyngeal testes are small and rounded (Figs 34-36), the follicles being arranged in two longitudinal rows. Yolk glands lie dispersed in the parenchyma. The ovaries are small and rounded. Judging from the histological condition of ovaries and testes, the holotype specimen is probably not fully mature.

The penis is provided with a spheroidal bulb and a penial papilla consisting of a turbinate basal part and a long, pointed tip (Figs. 39, 40). The muscular bulb houses the narrow, "T-shaped", anterior part of the seminal vesicle, receiving the separate openings of the sperm ducts at its anteroventral portion, while its posterodorsal part communicates with the irregularly shaped cavity in the base of the penial papilla. This plicate cavity in the turbinate part of the penis receives the numerous openings of penis glands and narrows abruptly to give rise to the narrow ejaculatory duct. The penis lumen is lined with a nucleated epithelium, underlain with a layer of circular muscle fibres.

The penis papilla is lined with a flat, nucleated epithelium and projects into the tubular canal of the penis sheath, or pseudophallus, the latter arising from the anterior section of the male genital antrum (Fig. 40). The canal of the penis sheath is lined with a nucleated epithelium and is surrounded by a thick, subepithelial layer of circular muscle, followed by a thin layer of longitudinal muscle. The penis sheath is covered with a relatively thick, nucleated epithelium that is underlain with a well developed, subepithelial layer of circular muscle and a layer of longitudinal muscle fibres. Furthermore, loosely arranged muscles fibres traverse the parenchyma of the free portion of the pseudophallus. In addition, a conspicuous annular zone of circular muscle extends from the base of the penis sheath well into the parenchyma of the body. Abundant erythrophilic penis glands open into the middle part of the male antrum.

The female copulatory apparatus is a large, spheroidal, moderately muscular organ provided with a female genital duct, receiving the openings of numerous erythrophilic glands. Many erythrophilic glands open into the beginning and especially into the posterior wall of the female genital duct. This female genital duct has many plicae and receives the separate openings of the ovovitelline ducts at its anterodorsal portion. The female genital duct is lined with a thick, nucleated epithelium and is surrounded by a

thick coat of muscle : a thin, subepithelial layer of longitudinal muscle, a relatively thick layer of intermingled circular and longitudinal fibres, and a third thin layer of longitudinal muscle fibres.



Figs 37-39. – *Novibipalium miyukiae*. ZMA V.Pl. 988.1. Sagittal sections of the copulatory apparatus; anterior to the right.

Discussion

In addition to the two new species of *Novibipalium* described in this paper three other species have been reported from Japan, viz. *N. falsifuscatum* Kawakatsu, Ogren & Froehlich, 1998, *N. trifuscostriatum* (Kaburaki, 1922), and *N. venosum* (Kaburaki, 1922) (cf. KAWAKATSU, 1991b; KAWAKATSU et al., 1998; KAWAKATSU & SASAKI, 2001; see also Table 2).

On the basis of its external appearance, with blackish brown colouration on the dorsal body surface and without stripes on both dorsal and ventral surface, *N. miyukiae* can easily be distinguished from *N. falsifuscatum* and *N. trifuscostriatum*.

N. venosum is a moderately sized species (2 specimens examined by the original author were 90 x 5 mm and 50 x 2.5 mm) with a lunate head with well-developed auricles, dark brown dorsal body surface, and without stripes on dorsal or ventral body surface. Both externally and anatomically, *N. miyukiae* and *N. venosum* are rather similar. As already pointed out by KAWAKATSU et al. (1991b), reidentification of KABURAKI'S (1922a) species presents serious difficulties. This is due to KABURAKI's classical descriptions and his rather simplified drawings of the copulatory apparatus. Muscle layers of the copulatory apparatus and details of glandular ducts are usually not detailed in his figures. Unfortunately, all of his original specimens were lost (cf. KAWAKATSU & SASAKI, 2004).

N. miyukiae is much smaller than *N. venosum*. Furthermore, the genital anatomy of the former differs from the latter in the following features : presence of a large, spherical penis bulb; penis papilla with turbinate base and long, pointed tip; penis lumen consisting of a T-shaped seminal vesicle, a middle section constituting a spacious bulbar cavity with many plicae, and a proximal section forming a tubular ejaculatory duct; the middle and posterior sections of the male antrum form a well-developed penis sheath provided with a long, wide and bellows-shaped copulatory canal that is surrounded by a thick muscle coat. Furthermore, in *N. miyukiae* abundant erythrophilic glands open into the posterior section of the penis lumen, while the female genital duct and the copulatory canal are very conspicuous.

The distance between the type localities of *N. miyukiae* and *N. venosum* is about 1000 km and crosses the Tsugaru Straits. The latter are also known as Blakiston's Line, representing one of the biogeographical boundaries of both plants and animals in Japan; the Strait opened some time during the Late Pleistocene, about 1 My ago (see KAWAKATSU et al., 1990). There is no indication that the Hakodate population of *N. miyukiae* is the result of an artificial introduction from Honshû.

In view of the arguments presented above, we conclude that *N. miyukiae* from southern Hokkaidô in northern Japan, represents a new species.

Novibipalium murayamai sp. nov.

Material : Holotype, ZMA V.Pl. 989.1, 3-6 June 1987, Sanjô City, Nîgata Prefecture, Chûbu Region, Honshû, sagittal sections on 46 slides (G).

Paratypes : ZMA V.Pl. 989.2, ibid., sagittal sections on 54 slides (G); V.Pl. 989.2, ibid., sagittal sections on 73 slides (PH); V.Pl. 989.2, ibid., sagittal sections on 66 slides (H); V.Pl. 989.2, ibid., transverse sections on 33 slides (PRE); NSMT 5419, ibid., sagittal sections on 60 slides (G); ZMA V.Pl. 989.3, ibid., whole mount of front end.

Other material examined : ZMA V.Pl. 990.1, 21 July 1987, Sanjô City, Nîgata Prefecture, Chûbu Region, Honshû, sagittal sections on 38 slides (PRE); V.Pl. 990.1, ibid., transverse sections on 30 slides (PRE); V.Pl. 990.1, ibid., sagittal sections on 42 slides (PG); V.Pl. 990.1, sagittal sections on 25 slides (T).

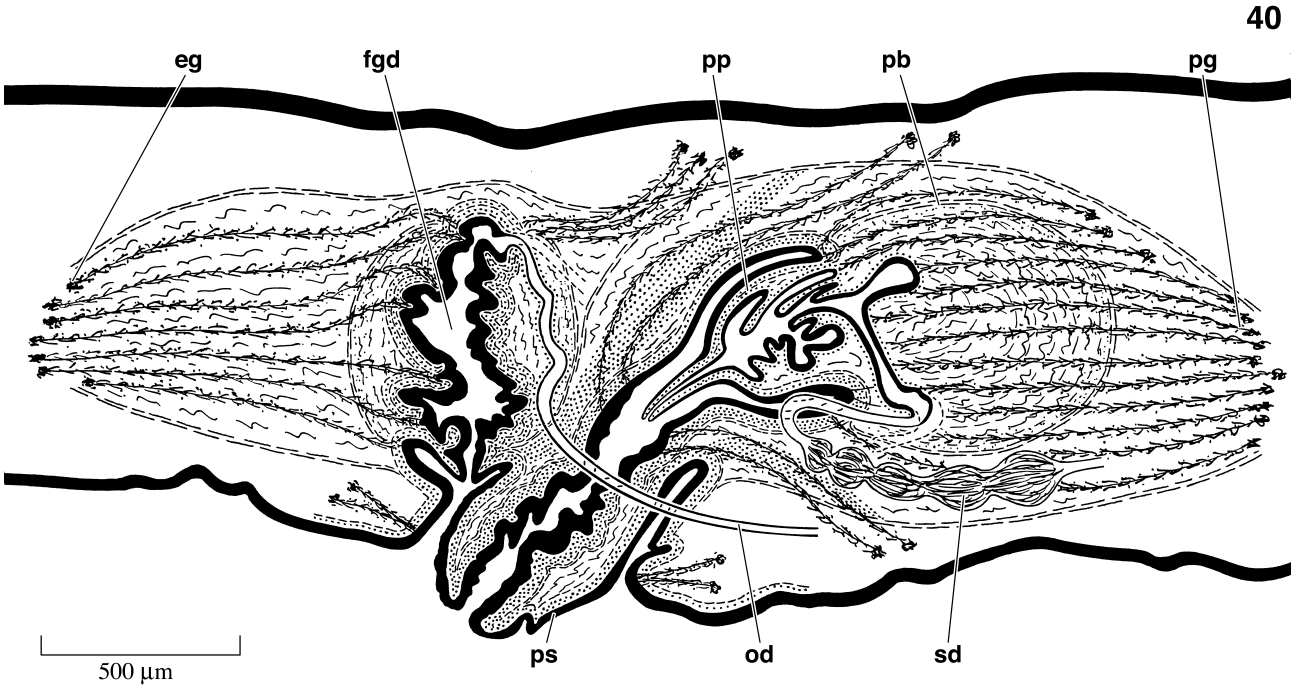


Fig. 40. – *Novibipalium miyukiae*. Holotype. ZMA V.Pl. 988.1. Sagittal reconstruction of the copulatory apparatus; anterior to the right.

Diagnosis

On external features *Novibipalium murayamai* sp. nov. can be distinguished from its congeners by the following features : moderate size (50 – 60 mm long), semilunar head with protruding, recurved auricles; head plate and auricles with yellowish brown border and crescent-shaped spots; uniform yellowish brown to ochre-coloured on the dorsal body surface, with a pair of brownish lateral stripes. The ventral surface is devoid of stripes. Anatomically the species is characterized by the following features : large, spherical penis bulb; short, conical penis papilla; sperm ducts opening into the mid-ventral portion of the intrabulbar, plicate seminal vesicle; male antrum giving rise to a highly muscular penis sheath, or pseudophallus; female genital duct with an obovoid dorsal section, receiving the separate openings of the ovovitelline ducts, and a bellows-shaped ventral part.

Ecology and distribution

Known only from the type locality, where it was collected from the garden of Mr. Kozakai's residence.

Etymology

The specific epithet is based on the family name of Mr. Hitoshi Murayama, whose cooperation for over 30 years has been invaluable for the turbellarian studies of the senior author.

Description

According to observations made by the collectors, the largest living, sexually mature specimen in elongated condition measured approximately 100 mm in length, with a body width of about 6 mm. Dimensions taken from three preserved specimens are as follows : TL : 56-60 mm; HML :27-32 mm; HGL : 35-40 mm; WM : 6-7 mm.

Figs 41-42. – *Novibipalium murayamai*. ZMA V.Pl. 989. Ventral (41) and dorsal (42) view of preserved specimen.

The wide, semilunate head provided with protruding and recurved auricles (Figs 41-42). The body is rather flat and broad, with only a slightly narrower "neck" region and bluntly pointed tail.

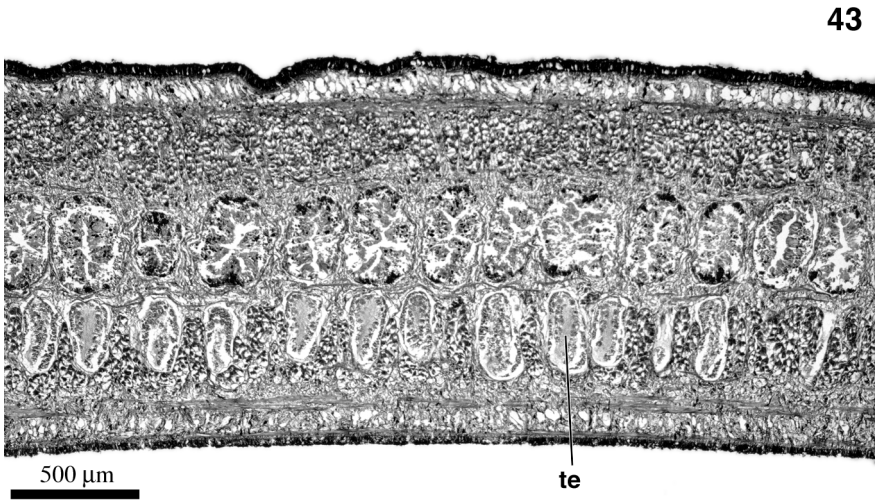


Fig. 43. – *Novibipalium murayamai*. ZMA V.Pl. 989.2. Sagittal section of the prepharyngeal region; anterior to the left; notice testes.

The dorsal surface is pale yellowish brown or ochre coloured, with the margins of the head plate and auricles being yellowish brown. The head pattern is very characteristic, consisting of several crescent-shaped spots arranged as follows : the outer, yellowish brown edge of the head plate, a crescent-shaped dark brown submarginal mark, a third, and crescent-shaped yellowish brown spot, followed lastly by a dark brown reniform spot on the "neck" or the basal part of the head plate. Two longitudinal brownish lateral stripes run from the "neck"region to the tail. The ventral surface is uniform greyish brown with relatively narrow creeping sole.

Numerous small eyes are located along the margin of the head and on the basal parts of the "neck".

The testes are located ventrally, extending from behind the ovaries to just posterior of the pharyngeal pouch (Fig. 43).

The penis consists of a large, spherical bulb and a conical papilla. The bulb houses a plicate cavity, receiving the separate openings of the sperm ducts at its mid-ventral portion. This intrabulbar cavity continues in the penial papilla as an undulating ejaculatory duct, opening at the tip of the papilla. The papilla is covered with a flat, nucleated epithelium, underlain with a relatively thick, subepithelial layer of circular muscle, followed by a thin layer of longitudinal fibres. The entire penis lumen (i.e. cavity and duct) is lined with a flat, nucleated epithelium, underlain with a thin, subepithelial layer of circular muscle and an equally thin layer of longitudinal muscle fibres.

The male genital antrum shows three sections : (a) an anterior, bowl-shaped cavity housing the penial papilla, (b) a middle, fusiform cavity, and (c) a posterior, wine glass-shaped part, opening into the male genital pore. The middle and posterior parts of the antrum form the lumen of a well-developed penis sheath, or pseudophallus. The lining epithelium of the anterior and middle sections of the antrum consists of relatively tall, nucleated, and highly glandular cells. The musculature around these parts consists of a thick, subepithelial layer of circular muscle and a much thinner layer of longitudinal muscle fibres; on the middle section the musculature becomes somewhat thicker than on the other sections. Middle and posterior section of the antrum are well separated by a conspicuous diaphragm; the posterior part is lined with a flat, nucleate and glandular epithelium that is underlain with thin layers of circular and longitudinal muscles (Fig. 44).

The penis lumen and the anterior and middle portions of the male antrum receive the abundant secretion of erythrophilic penis glands.

The free portion of the penis sheath is covered with a flat, nucleated and glandular epithelium, underlain with thin layers of circular and longitudinal muscle. Loosely arranged muscles lie dispersed in the parenchyma of the penis sheath (Fig. 45).

The female genital duct comprises two parts, viz. an obovoid dorsal section receiving the separate openings of the ovovitelline ducts, and a ventral, bellows-shaped section opening into the anteroventral part of the common genital antrum. The female genital duct is lined with a thick, nucleated epithelium that is pierced by the openings of numerous eythrophilic glands. The dorsal part of the duct is surrounded by a thick, subepithelial layer of circular muscle, followed by a thin layer of longitudinal fibres. The muscle coat around the ventral part of the female genital duct is much thinner.

The common genital antrum is lined with a flat, glandular and nucleated epithelium.

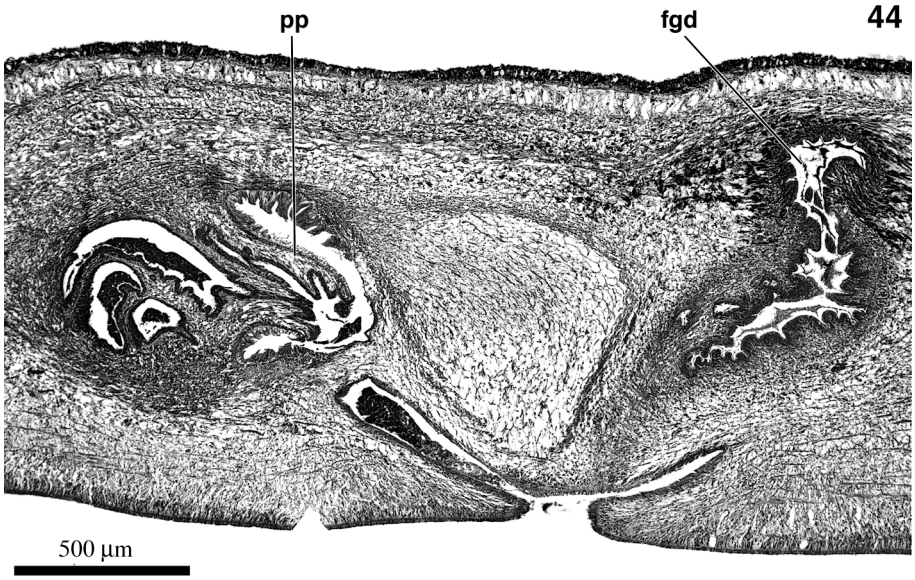


Fig. 44. – *Novibipalium murayamai*. NSMT 5419. Sagittal section of the copulatory apparatus; anterior to the left.

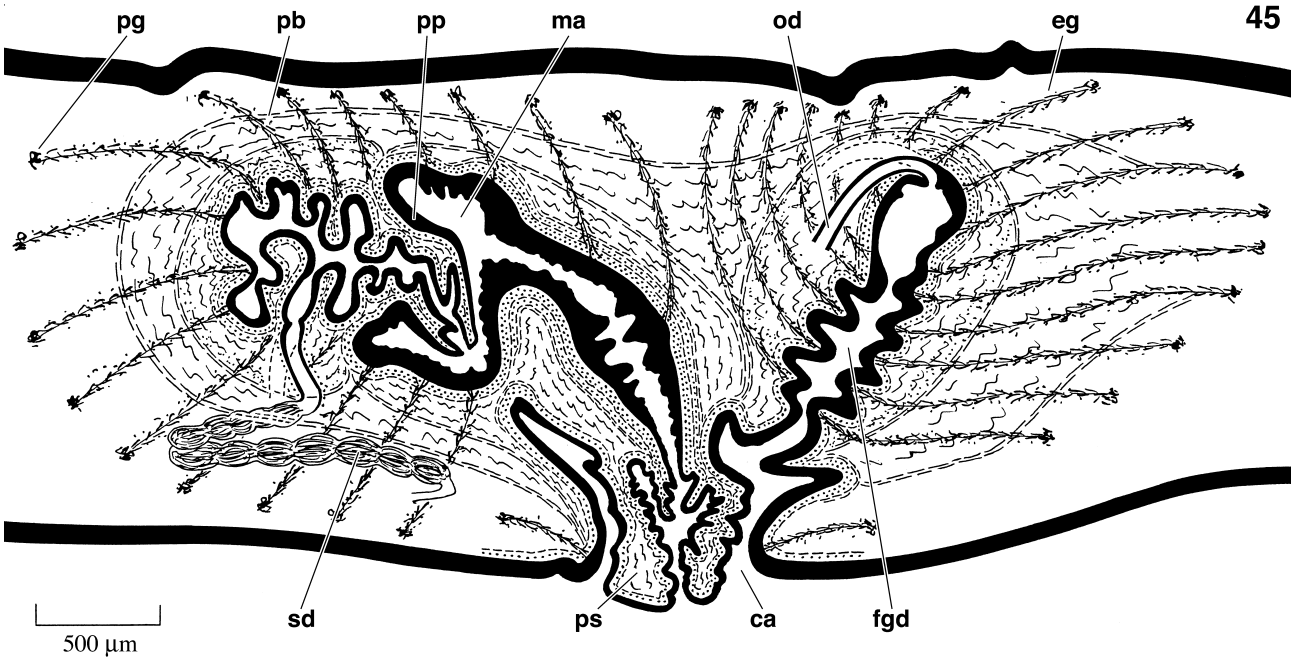


Fig. 45. – *Novibipalium murayamai*. Holotype. ZMA V.Pl. 989.1. Sagittal reconstruction of the copulatory apparatus; anterior to the left.

Karyology

Chromosome complement : 2x = 10, with a karyotype of 2m + 2sm + 2sm + 2sm + 2m (cf. KAWAKATSU et al., 1990, figs 15C, 16 : *Bipalium* sp. 2; OKI et al., 1991, fig. 3 : *Bipalium* sp. 2).

Discussion

N. murayamai can be distinguished from the other four Japanese species of *Novibipalium* by its characteristic external features : lunate or semilunate head with moderately protruding, recurved auricles; head plate and auricles provided with a conspicuous crescent-shaped mark; a pair of brownish and rather broad longitudinal stripes on a yellowish brown dorsal surface; lack of stripes on the ventral body surface.

Among the known species of *Novibipalium*, the mid-ventral openings of the sperm ducts into the bulbar cavity are conspicuous only *N. murayamai*. Another unique character of *N. murayamai* is the presence of a well-

developed, highly muscular penis sheath, with its middle and posterior sections being separated by a conspicuous diaphragm. With respect to the female reproductive apparatus *N. murayamai* can be distinguished by its large female genital duct consisting of a wide cavity, followed by a bellows-shaped section that communicates with the anteroventral part of the common genital antrum.

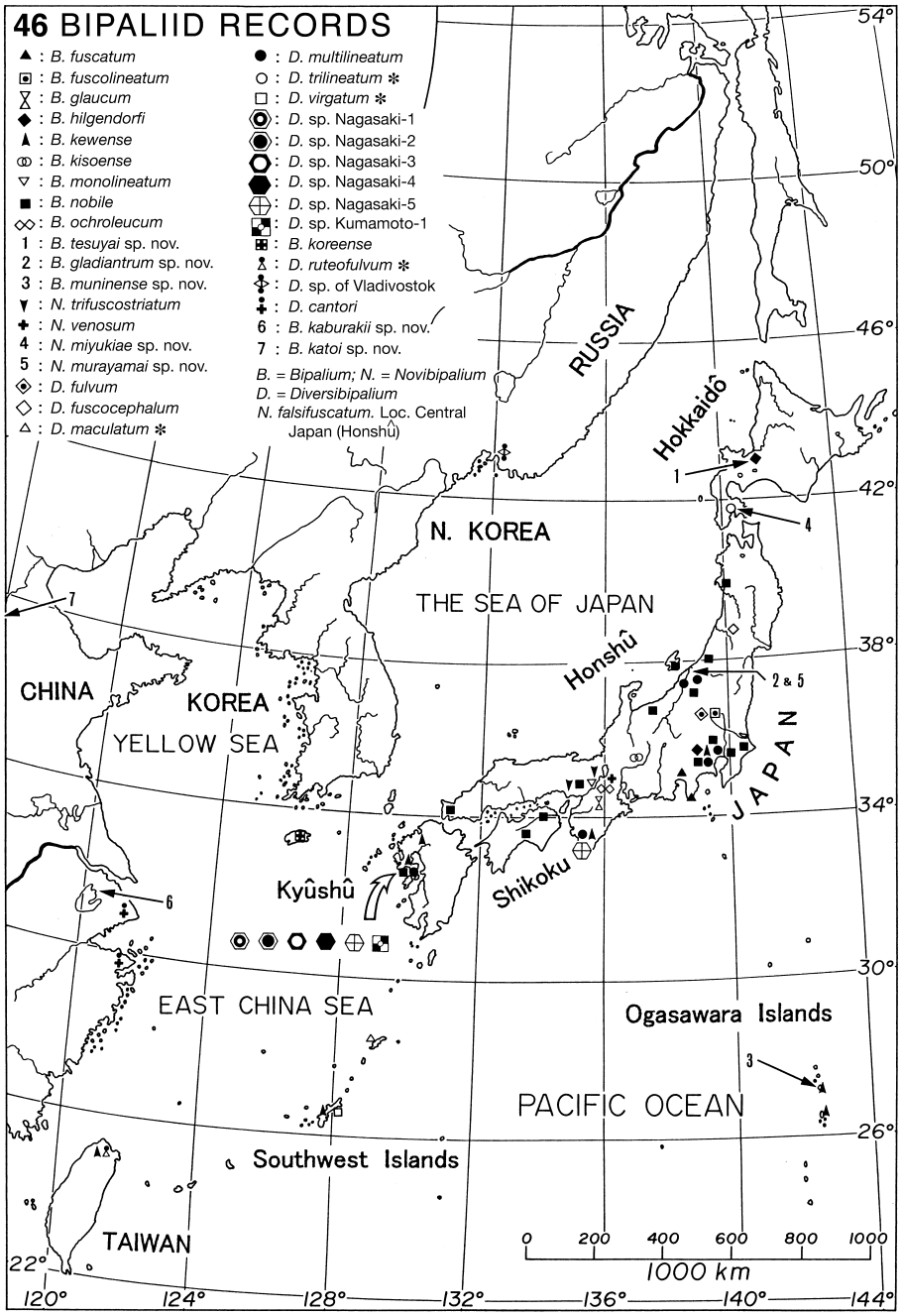


Fig. 46. – Distributional records of bipaliid species from the Far East. Each symbol on the map generally represents a separate record. Arrows with the numbers 1 – 5 point to the type localities of the five new Japanese species described in this paper; numbers 6 – 7 point to the localities of the two new Chinese species. N.B. The locality of *N. falsifuscatum* is only known as Central Japan.

BIOGEOGRAPHIC OVERVIEW OF FAR EASTERN SPECIES, WITH TAXONOMIC NOTES

Japan

A total of 18 bipaliid species has been reported from Japan (Tables 1-3, Fig. 46) : nine *Bipalium* species, three *Novibipalium* species, and six species of the collective group *Diversibipalium*. Additonally, six unidentified species, tentatively considered *Diversibipalium* species, are known from the vicinity of Nagasaki City, Kyûshû, in South Japan (KAWAKATSU et al., 2000; YAMAMOTO et al., 2001, 2003). Furthermore, two *Diversibipalium* spp. have been recorded also from Central Japan. With respect to these species, the following unresolved taxonomic problems remain.

1. Of the nine species of *Bipalium*, four have not yet been redescribed from a modern taxonomic point of view : *B. fuscolineatum* Kaburaki, 1922; *B. glaucum* (Kaburaki, 1922); *B. kisoense* Kaburaki, 1922; *B. ochroleucum* Kaburaki, 1922. There is a possibility that some of these four nominal species constitute synonyms.

2. A comparative taxonomic study, based on new material, of *Novibipalium falsifuscatum* Kawakatsu, Ogren & Froehlich, 1998 and *N. venosum* Kawakatsu, Ogren & Froehlich, 1998 is necessary since KABURAKI (1922a) gave a mixed description of the two species under the name of a single species, viz. "*Placocephalus fuscatus* Stimpson". Later, this species was separated into two species, viz. *Bipalium fuscatum* Stimpson, 1857 and *Novibipalium falsifuscatum* Kawakatsu, Ogren & Froehlich, 1998, with the latter being classified only on the basis of KABURAKI'S (1922a) description with illustrations of the copulatory apparatus (cf. KAWAKATSU, OGREN & FROEHLICH, 1998).

TABLE 1

Comparative morphological and anatomical data for Japanese species of *Bipalium*. Dimensions of the animals shown as length x width. Abbreviations and conventions for stripes : md, mid-dorsal stripe; la, lateral stripes; mg, marginal stripes (occurring only on the prepharyngeal region of the body). Chromosome data : [1] 2x=18. KAWAKATSU et al. (1990, 2000); OKI et al. (1991, 1995); YAMAMOTO et al. (2001, 2003); [2] 2x=10. KAWAKATSU et al. (1990, 2000); OKI et al. (1991, 1995); YAMAMOTO et al. 2001, 2003); [3] 2x=10. KAWAKATSU et al. (1990 : *Bipalium* sp. 3); OKI et al. (1991 : *Bipalium* sp. 3).

SPECIES		*B. fuscatum*	*B. fuscolineatum*	*B. glaucum*	*B. hilgendorfi*	*B. kewense*	*B. kisoense*	*B. monolineatum*	*B. nobile*	*B. ochroleucum*	*B. tetsuyai*	*B. glandiantrum*	*B. muninense*
BODY	Size (in mm)	80-120 x 7-8	50 x 3	120-200 x 3.5-4	60-80 x 5	120-200 x 5	30-35 x 2	35 x 5	300-1000 x 5-7	50 x 3.5	60 x 2.5	30-50 x 5	50 x 3-4
HEAD	Plate	lunate	lunate	rotundate	rotundate	rotundate	lunate	lunate	rotundate	rotundate	lunate	semilunate	lunate
	Auricle	large	large	short	short	short	moderate	well developed recurved	short	short	moderate	moderate	moderate
DORSAL SIDE	Ground colour	black	dark olive	greenish grey	reddsh brown	yellowish brown	dark olive brown	dark brownish orange	yellowish brown	dark yellow	brown	yellowish brown	dark brown
	Stripes	–	1md	–	1md	1md + 2la + 2mg	1md	1md	1md + 2la + 2mg-pre	1md	1md	1md + 2la	1md + 2la + 2mg
	Patterns	–	–	–	–	wide patch (neck)	–	–	–	–	h.p. with dark edge	–	–
VENTRAL SIDE	Ground colour	dark grey	grey	pale	pale	pale	pale brown	pale	pale	pale	pale	pale brown	pale
	Stripes	–	–	–	–	2la	–	–	2la	–	–	2la	–
	Patterns	–	–	–	–	wide patch (neck)	–	–	–	–	narrow patch (neck)	dark colour (neck)	–
PENIS	Penis bulb	large	moderate	large	moderate	large	moderate	moderate	large	moderate	large	moderate	moderate
	Penis papilla	small	moderate	large, long	moderate	moderate	large, long	large, long	moderate	large, long	moderate	large, long	large
	Bulbar cavity	narrow	moderate	narrow	wide	wide	narrow	narrow	wide	moderate	wide	narrow, tubular	wide
	Ejaculatory duct	short, tubular	short, tubular	short, tubular	moderate, tubular	long, tubular	moderate, tubular	long, tubular	long, tubular	narrow, tubular	wide	long, tubular	wide
MALE ANTRUM	Antrum	moderate	moderate	moderate	moderate	wide	moderate	moderate	moderate	moderate	wide	moderate	wide, tubular
	Penis sheath	–	–	–	–	–	–	–	–	–	–	–	–
FEMALE COP. APP.	Organ	moderate, muscular	small	small	moderate	large	moderate	moderate	moderate	moderate	moderate	moderate	moderate
	Female genital canal	narrow, tubular	narrow	narrow, tubular	wide	wide, tubular	narrow, tubular	narrow, tubular	moderate, tubular	moderate, tubular	moderate	wide	wide
COMMON GENITAL ANTRUM		moderate	narrow	narrow	wide, glandular	wide, glandular	moderate	moderate	moderate	wide	moderate	moderate, glandular	moderate
CHROMOSOME NUMBER (2 x)		?	?	?	?	18 [1]	?	?	10 [2]	?	?	?	10 [3]

TABLE 2

Comparative morphological and anatomical data for Japanese species of *Novibipalium*. For further explanation, see Table 1. [4] 2x = 10. KAWAKATSU et al. (1987 : *Bipalium* sp. TFS type); OKI et al. (1988 : *Bipalium* sp. TFS type); [5] 2x = 10. KAWAKATSU et al. (1990 : *Bipalium* sp. 2); OKI et al. (1991 : *Bipalium* sp. 2).

SPECIES		*N. falsifuscatum*	*N. trifusco-striatum*	*N. venosum*	*N. miyukiae*	*N. murayamai*
BODY	Size (in mm)	120 x 4	55 x 6-8	50-90 x 2.5	20-26 x 3	50-60 x 6-7
HEAD	Plate	lunate	lunate	lunate	rotundate	lunate (crescent mark)
	Auricle	well developed	short	well developed	moderate	moderate
DORSAL SIDE	Ground colour	black	dark brown	dark brown	blackish brown	yellowish brown
	Stripes	–	1md + 2la	–	1md	2la
	Patterns	–	–	–	–	h.p. with brown edge
VENTRAL SIDE	Ground colour	dark grey	pale	pale	greyish brown	greyish brown
	Stripes	–	–	–	–	2la
	Patterns	–	–	–	–	–
PENIS	Penis bulb	large	large	small	large	large
	Penis papilla	short	short	moderate	moderate	large
	Bulbar cavity	narrow, tubular	wide	moderate	wide	wide
	Ejaculatory duct	short, wide	short, tubular	short, wide	long, tubular	long, tubular
MALE ANTRUM	Antrum	moderate	moderate	moderate	moderate	moderate
	Penis sheath	well developed	well developed	well developed	well developed	well developed
FEMALE COP. APP.	Organ	large	large	small	moderate	large
	Female genital canal	wide	wide	wide, tubular	wide	wide, tubular
COMMON GENITAL ANTRUM		shallow	shallow	wide	shallow	moderate
CHROMOSOME NUMBER (2 x)		?	10 [4]	?	?	10 [5]

3. Proper reidentification of STIMPSON's species *Diversibipalium maculatum* (Stimpson, 1857), *D. trilineatum* (Stimpson, 1857), and *D. virgatum* (Stimpson, 1857) is not possible, due to their very superficial original descriptions that lack any illustrations. Therefore, these three species can be classified only as "species inquirendae."

4. The descriptions of *Diversibipalium fulvum* (Kaburaki, 1922) and *D. fuscocephalum* (Kaburaki, 1922) are based on non-sexual specimens, albeit supplied with colour sketches of the external appearance of the animals. Therefore, these species can be identified properly only after new material of sexual specimens has become available. Thus, both species must be considered as a "species incertae sedis."

5. Although sexual specimens of *Diversibipalium multilineatum* (Makino & Shirasawa, 1983) have not yet been obtained, the external features of the species seem to be sufficiently different from other Japanese bipaliids to consider it as a separate "species incertae sedis."

Korea

Only a single species has been reported from Quelpart Island, viz. *Bipalium koreense* Frieb, 1923 (Table 4, Fig. 47). This moderately large species (77-81 mm in length, with a width of 9 mm, in preserved condition) has been described with yellowish brown to greyish yellow dorsal surface, devoid of stripes; ventral side pale and also devoid of stripes (cf. FRIEB, 1923, pl. 15, fig. 1). The species shows a large penis bulb with a narrow cavity, and a very long, pointed penis papilla (being coiled in Frieb's figure) with a tubular ejaculatory duct. The female genital duct is small and tubular (cf. FRIEB, 1923, T. 15, figs 2-3; Fig. 47).

B. koreense shows some resemblance, both externally and anatomically, to *B. glaucum* (Kaburaki, 1922) from Japan (cf. KABURAKI, 1922a, figs 20-21, pl. 1, fig. 17). Both species are known only from their original descriptions.

According to notes made by RS in October 1989, the catalogue of the Natural History Museum, London, lists spirit specimens as types.

Taiwan

Two species have been reported from Taipei and its surroundings (Table 4, Fig. 40) : *Bipalium kewense* Moseley, 1878 (synonym *"Placocephalus virgatus* (Stimpson)" sensu Kaburaki, 1922; cf. WINSOR, 1983; KAWAKATSU & SASAKI, 2001), and *Diversibipalium ruteofulvum* (Kaburaki, 1922). A colour sketch of the last-mentioned species was given by KABURAKI (1922a, Pl. I, fig. 10) and it is here considered to be a "species incertae sedis".

For the latest taxonomic and distribution data of land planarians from Taiwan, see KAWAKATSU et al. (2005) and WU et al. (2005).

China

Taxonomic reviews of the bipaliids from eastern and south-eastern China can be found in KAWAKATSU & LUE (1984), LUE & KAWAKATSU (1986), OGREN & KAWAKATSU (1987, 1988), and KAWAKATSU (1991b). Discussions on the occurrence of *Bipalium nobile* Kawakatsu & Makino, 1982 and *Bipalium kewense*

TABLE 3

Comparative morphological and anatomical data for Japanese species of *Diversibipalium.* * : species inquirenda. For further explanation, see Table 1. [6] 2x=10. KAWAKATSU et al. (2000); KUBOTA et al. (2001); YAMAMOTO et al. (2001, 2003); [7], [8], [9], [10] 2x=10. KAWAKATSU et al. (2000); YAMAMOTO et al. (2001, 2003); [11] 2x=12. KAWAKATSU et al. (2000); KUBOTA et al. (2001); YAMAMOTO et al. (2001, 2003); [12] 2x=10. YAMAMOTO et al. (2003).

SPECIES		*D. fulvum*	*D. fuscocephalum*	*D. multilineatum*	*D. maculatum* *	*D. trilineatum* *	*D. virgatum* *	*D.* sp. Nagasaki-1 (Isahaya)	*D.* sp. Nagasaki-2 (Shimabara)	*D.* sp. Nagasaki-3 (Shimabara)	*D.* sp. Nagasaki-4 (Nagasaki)	*D.* sp. Nagasaki-5 (Shimbara)	*D.* sp. Kumamoto-1 (Kuamoto)	*D.* sp. Chiba City-1	*D.* sp. Chiba City-2
BODY	Size (in mm)	20 x 7.5	35 x 3	100-200 x 5	75 x 5	40 x 5.5	50 x 4	70 x 4	70 x 6	80 x 5-7	30x 2-4	200 x 10-15	30 x 3	96 x 5	160 x 5
HEAD	Plate	rotundate	lunate	rotundate	lunate	lunate	lunate	lunate	lunate	semilu-nate	lunate	lunate	lunate	lunate	lunate
	Auricle	inconspi-cuous	moder-ate, recurved	short	large	moderate	large, recurved	moderate	well devel-oped	well devel-oped	moderate	moderate	moderate	short	moderate
DORSAL SIDE	Ground colour	pale red-dish yel-low	dark brown	yellow-ish brown	dark brown	pale yel-lowish brown	pale orange	dark brown	dark greyish brown	light yel-lowish brown	dark brown	blackish brown	dark blackish brown	dark yel-lowish brown	dark yel-lowish brown
	Stripes	–	1md	1md + 2la	–	3 lines	5 lines	1md + 2la	1md + 2mg	1md + 2mg	1md	–	–	1md + 2la	–
	Patterns	–	–	–	**–?**	–	–?	–	–	–	–	pig-mented	–	–	–
VENTRAL SIDE	Ground colour	pale	pale	pale	?	?	?	pale	pale	pale	pale	pale	blackish brown	pale	pale
	Stripes	**–?**	–?	1md + 2la	?	?	?	2la	2la	2la	–	–	–	–	–
	Patterns	–?	**–?**	–	**?**	?	?	–	–	–	–	pig-mented	–	–	–
PENIS	Penis bulb	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Penis papilla	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Bulbar cavity	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Ejaculatory duct	?	?	?	?	?	?	?	?	?	?	?	?	?	?
MALE ANTRUM	Antrum	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Penis sheath	?	?	?	?	?	?	?	?	?	?	?	?	?	?
FEMALE COP. APP.	Organ	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	Female genital canal	?	?	?	?	?	?	?	?	?	?	?	?	?	?
COMMON GENITAL ANTRUM		?	?	?	?	?	?	?	?	?	?	?	?	?	?
CHROMOSOME NUMBER (2 x)		?	?	10 [6]	?	?	?	10 [7]	10 [8]	10 [9]	10 [10]	12 [11]	10 [12]	?	?

Moseley, 1878 can be found in KAWAKATSU et al. (2001); see also SASAKI et al. (2001). Following up on these studies, OGREN et al. (1997) listed three species for NE China : *Bipalium cantori* (Wright, 1860), *B. grayi* (Wright, 1860), *B. kewense*.

We have made a careful re-examination of the descriptions and published records for China and came to the conclusion that (1) two species should be placed in the collective group *Diversibipalium*, (2) presumed "*B. cantori*" from Shanxi Province (cf. KATÔ, 1950) represents a new species (see above), and (3) presumed "*B. cantori*" from Chiangsu Province (cf. KABURAKI, 1922b) also represents a new species (see above). Our arguments concerning *B. grayi* and *B. cantori* are detailed below.

Diversibipalium grayi (Wright, 1860) comb. nov. Species inquirenda

WRIGHT (1860) described this species under the name of *Dunlopea Grayia*. His figure of the animal is based on a "coloured drawing by Dr. Cantor (the collector of the animal) in the collection of the British Museum". Judging by both textual description and figure (for a reproduction of Wright's text and figure, see KAWAKATSU et al., 2001), the species is characterized by a triangular head with pointed, recurved auricles, brownish yellow ground colour, dark stripe along the margin of the head, and two broad, dark longitudinal stripes, perhaps flanked by a thin stripe on either side (Table 4). Since the genital anatomy of the species is not known, it is here classified under *Diversibipalium*.

Diversibipalium cantori (Wright, 1860) comb. nov. (Figs 27-29)

The species was described by WRIGHT (1860) and VON GRAFF (1899, T. XIII, figs 1-2), the last-mentioned worker basing his description on the original specimens, as a large species (140 - 200 mm in length with a width of 6 - 8 mm, in preserved condition) showing the following features : an "expanded hammer-head-like" with recurved auricles; a dark brown edge surrounding the head plate; a dirty yellow dorsal surface showing many small, dark spots; a thin, dark mid-dorsal stripe; two dark lateral stripes that expand on the head to form very clear beak-shaped spots; two dark marginal stripes; devoid of stripes in the tail region; ventral surface of the head provided with pale brown edge and body surface showing two dark marginal stripes (Table 4).

Since the genital anatomy of the species is not known, we do here tentatively place the species in the collective group *Diversibipalium;* it is considered to be a "species incertae sedis." The animal was reported from Ningpo (29º45' N 121º33'E), Chekiang (=Zhèjiang) Province, SE China.

According to notes made by RS in October 1989, the catalogue of the Natural History Museum, London, lists the presence of the preserved type specimen.

Primorskiy (Russia)

A single, non-sexual specimen was described as *Bipalium* sp. from Vladivostok. Details on the external features of this specimen can be found in KAWAKATSU et al. (2000) and SASAKI et al. (2001). In this paper we classify this animal as *Diversibipalium* sp. of Vladivostok (Table 4); it is a "species incertae sedis."

TABLE 4

Comparative morphological and anatomical data for *Novibipalium, Bipalium*, and *Diversibipalium* species from SE Asia. * : species inquirenda. For further explanation, see Table 1.

COUNTRIES		**INDO-NESIA**	**KOREA**	**TAIWAN**		**CHINA**				**RUSSIA**
SPECIES		***N. alteri-fuscatum***	***B. koreense***	***B. kewense***	***D. ruteo-fulvum***	***B. kaburakii***	***B. katoi***	***D. cantori***	***D. grayi****	***D.* sp. Vladivostok**
BODY	Size (in mm)	120 x 4	77-81 x 9	60 x 3	45 x 6	165 x 5	70 x 4	140-200 x 6-8	?	20 x 4.8
HEAD	Plate	lunate	lunate	rotundate	lunate	semilunate	rotundate	lunate	lunate	lunate
	Auricle	well developed, recurved	moderate	short	moderate	moderate?	short	moderate	recurved	short?
DORSAL SIDE	Ground colour	black	yellowish-greenish brown	pale yellow	dark reddish brown	dark brown	blackish brown	yellowish brown	yellowish brown	pale greyish
	Stripes	–	–	1md + 2la + 2mg	–	1md + 2la + 2mg	1md + 2la + 2mg	1md + 2la + 2mg	1md? + 2la	–
	Patterns	–	–	wide patch (neck)	–	–	head with arched dark edge	2 beak-shaped patterns	?	–
VENTRAL SIDE	Ground colour	dark grey	pale	pale?	pale	pale	pale?	pale	?	pale
	Stripes	–	–	2la?	–	–	–	–	?	–
	Patterns	–	–	wide patch (neck)?	–	–	–	–	?	–
PENIS	Penis bulb	moderate	globose, large	large?	?	moderate	ellipsoidal, large	?	?	?
	Penis papilla	small	very long (coiled)	moderate?	?	moderate	short	?	?	?
	Bulbar cavity	moderate	narrow	wide?	?	moderate	wide	?	?	?
	Ejacula-tory duct	wide	long, tubular	long, tubular?	?	moderate, tubular	wide	?	?	?
MALE ANTRUM	Antrum	moderate	moderate	wide?	?	moderate	moderate	?	?	?
	Penis sheath	well developed	–	–	?	–	–	?	?	?
FEMALE COP. APP.	Organ	moderate	small	large?	?	small	small	?	?	?
	Female genital canal	wide	moderate, muscular	wide, tubukar?	?	moderate	moderate	?	?	?
COMMON GENITAL ANTRUM		wide	moderate	wide, glandular?	?	narrow	narrow	?	?	?
CHROMOSOME NUMBER (2 x)		?	?	18?	?	?	?	?	?	?

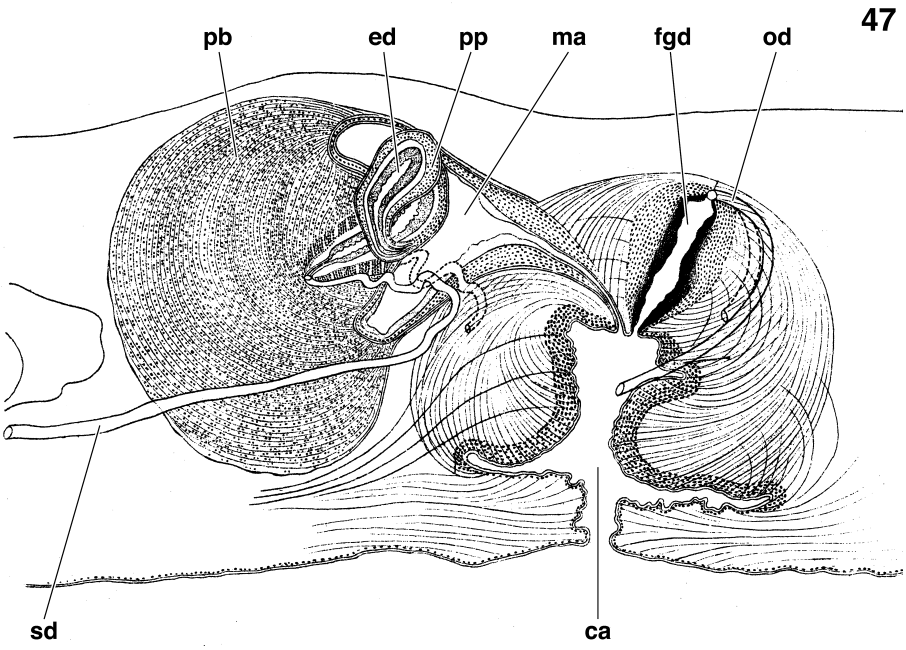


Fig. 47. – *Bipalium koreense*. Sagittal reconstruction of the copulatory apparatus (after Frieb, 1923 ; retouched and labels adapted according to current terminology) ; anterior to the left.

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SHORT NOTES

B chromosomes and asymmetry of eye lenses in the yellow-necked mouse, *Apodemus flavicollis* (Rodentia, Mammalia)

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KEY WORDS : *Apodemus flavicollis*, B chromosomes, eye lens, asymmetry.

Dry eye lens weight, estimated by method of LORD (1), is one of the best parameters for assessing the age of specimens of rodent species. Due to the absence of blood vessels in the lens, variations caused by physiological and environmental changes are less pronounced than in other (phenotypic) traits. Another important feature is that the weights of the eye lens proceed to increase after attainment of sexual maturity (2). According to DAPSON & IRLAND (3) the accumulation of the soluble fraction of tyrosine in the lens is representative of growth, whereas the conversion of the soluble to the insoluble fraction is regarded as aging. The pace of growth matches the logarithm of age. By measuring the dry lens weight of animals of known age, a growth curve that can be used to estimate the age of specimens can be easily obtained. Such a curve can be of great use in population studies of rodents.

All populations of the yellow-necked mouse, *Apodemus flavicollis,* studied so far are characterized by different B chromosome frequencies. B chromosomes are dispensable supernumerary chromosomes that do not recombine with A chromosomes. B chromosomes evolve independently of the standard chromosome complement (4). The effects of B chromosomes are numerous but it is difficult, with minor exceptions, to follow them at the phenotype level. They differ from one species to another. Developmental effects of B chromosomes are not rare in plants and are occasionally observed in animals. A good example is the grasshopper, *Myrmeleotettix maculatus* (5, 6). In *A. flavicollis* relationships between some morphometric characteristics are changed in the presence of Bs (7).

Measurement of asymmetry of morphometric characteristics is frequently used to study the effects of environmental and genetic factors (8). In this study variations of lens asymmetry relative to the mass of the lenses (i.e. the age of the specimen) were studied in the context of the presence of B chromosomes in populations of *A. flavicollis* with the aim to establish do the presence of Bs influences that relation.

Samples of the yellow-necked mouse *Apodemus flavicollis* were collected from three localities in Yugoslavia. These were Mt. Cer (CQ84), Mt. Avala (DQ64) and Mt. Fruška Gora (DR00) from 1993-1995, and from 1997-2000 (UTM coordinates are in brackets). A total of 133 animals were collected (Cer n=54, Avala n=48, Fruška Gora n=31). Chromosomes were prepared from bone marrow according to the standard procedure (9). The number of chromosomes was defined by scoring 30 metaphases per animal. The number of animals with B chromosomes per population was expressed as a frequency designated as fB. Eyeballs were removed and stored in 10% formaldehyde. After two weeks the lenses were dissected from the eyeballs, cleaned, rinsed with distillated water and dried in an oven at 80ºC for 48 h (10). Immediately after removal from the oven, they were weighed to an accuracy of 0.01 mg using a Mettler laboratory scale. Each lens (l1, l2) of an individual specimen was weighed separately, but without knowledge about left or right origin. The mass of a pair of lenses was used as an age indicator. Weighing of the lenses was performed by the same person (M.V.), with double-checking, without any prior knowledge of the results of chromosome analyses. The differences between pairs of lenses (asymmetry) were calculated as abs(l1-l2). Correlation analysis and Student t-test were performed using Stat 5.0 software.

The frequencies of animals with Bs were similar at the Cer and Avala (0.37 and 0.35) localities, while at Fruška Gora the frequency was higher (0.45), with average frequency 0.38. Dry eye lens weight measurements revealed that the population of specimens from Fruška Gora was the youngest, whereas the population from Avala was the oldest (Table 1). Samples from all three localities were pooled in order to analyze the effects of B chromosomes. Sample of animals with Bs was mostly made of animals with 1B (68.6%) and the rest was made of animals with 2B (19.6%), 3B (9.8%) and 4B (2%). Due to the small number of animals with more than one B we analyzed only the effects of presence of Bs. In pooled samples the ratio of males to females was 1 :1.38. Absolute differences in lens masses ranged from 0.1 to 3.1 mg, representing 0.29-21.65% of the weight of the lens. Estimated measurement error does not exceed significantly the mean value of asymmetry. Taking into consideration

these variations of lens mass, the correlation coefficients between the total weight of both lenses (age) and the absolute asymmetry were calculated for animals with and without B chromosomes (Fig. 1).

TABLE 1.

Weights of dry eye lenses (in mg) ±SE in males (M) and females (F) without (B0) and with (B+) B chromosomes (n – number of animals, fB – frequency of animals with Bs).

Locality	B0			B+			
	F	M	n	F	M	n	fB
Mt. Cer	23.9 ±1.17	24.7 ±1.58	34	24.3 ±1.56	20.9 ±2.19	20	0.37
Mt. Avala	22.7 ±1.80	25.5 ±2.40	31	24.4 ±1.73	26.6 ±2.69	17	0.35
Mt. Fruška Gora	20.4 ±2.39	22.1 ±2.91	17	20.7 ±2.36	20.4 ±1.47	14	0.45
Total	23.5 ±0.80		82	22.4 ±0.80		51	0.38

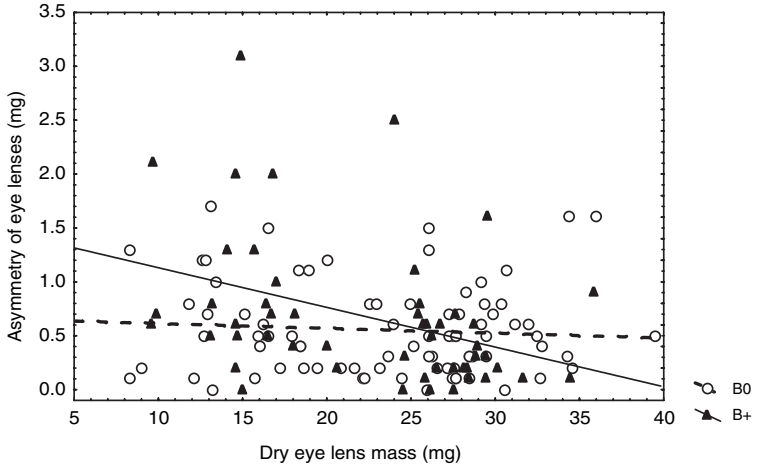

Fig. 1. – Correlation between dry eye lens mass (in mg) and lens asymmetry (abs(l1-l2)) in the group of animals without (B0) and with (B+) B chromosomes

The aim was to see whether the level of asymmetry of the lens was age-dependent in these two groups. It appears that in the group of animals without Bs (B0), the age and asymmetry of eye lenses were not dependent (r=0.08), while in the group of animals with Bs (B+) the correlation was significant and with a negative sign (r=-0.38). Results of t-test (t=-2.88, p=0.006) approve that the correlation coefficient differs significantly from zero. Therefore, in the last group the asymmetry decreased with age. It is possible that B chromosome carriers with a high degree of asymmetry were eliminated from populations at a higher rate. Previously it was shown that the presence of Bs had an effect of prolonging the mitotic cycle in the plant *Lolium perenne* (11). It is possible that the presence of Bs affected early embryonic and postnatal development. GLIWICZ & JANCEWICZ (12) proposed that significant differences in weight between left and right lenses in specimens of *Sorex minutus* are the consequence of illness. It is possible that weight differences between left and right lenses could be ascribed to different diseases and developmental disturbances. If differences in the weights of the lenses lead to anomalies in vision (which is very important for *A. flavicollis* as they are mostly nocturnal animals), it can be expected that selection pressure will be very high in animals that have such disorders. But differences in dry weight between lenses could be also produced by differences in developmental stability between groups of animals with and without Bs.

In the population from Mt. Jastrebac, BLAGOJEVIĆ & VUJOŠEVIĆ (13) found that under conditions of stress due to overcrowding, young animals with Bs were preferentially eliminated. Furthermore, it was found that B chromosomes in this species were characterized by the presence of specific molecular marker sequences (14). The results of this study imply that the presence of B chromosomes influences relation between lens asymmetry and age, although the causative role of Bs remains to be established.

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Leptosynapta minuta (Becher, 1906) (Echinodermata, Holothuroidea), a new record for Belgian marine waters

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ABSTRACT. *Leptosynapta minuta* Becher, 1906 is described for the first time from Belgian marine waters. This brings a new order (Apodida), family (Synaptidae), subfamily (Leptosynaptinae) and genus (*Leptosynapta*) to the Belgian holothuroid fauna. A morphological description of the specimens, the habitat characteristics and all literature records with a distribution map of *L. minuta* is given.

KEY WORDS : Apodida, *Leptosynapta minuta*, new record, Belgium, distribution.

INTRODUCTION

Until now only four holothuroid species were known from Belgian marine waters (MASSIN, 1988; MASSIN & DE RIDDER, 1989), all belonging to the order Dendrochirotida. The holothuroid fauna in adjacent countries is much richer (VANDEN BERGHE & APPELTANS, 2003), including seven apodid species in France of which at least five also occur in the North Sea (MADSEN & HANSEN, 1994; HANSSON, 2001), at times very close to the Belgian border (VAN DAMME & HEIP, 1976). This supposed absence of apodids from Belgian marine waters is surprising because European apodids are known to live in sandy sea beds (KOEHLER, 1924; MORTENSEN, 1927; CHERBONNIER, 1953; PICTON, 1993), the dominant biotope along our coast. The present record fills this gap.

MATERIAL AND METHODS

The sampling station where *Leptosynapta minuta* was found is situated on the northern side of the Kwintebank (51° 18,65; 2° 40,75 (WGS84)), in the Flemish bank area. This station was investigated during a sampling expedition on 13 February 2003, using the research vessel 'Zeeleeuw'. The sample was taken with a Van Veen grab (sampling surface area : 0.1026 m²) and sieved after fixation (with 8% formaldehyde-seawater solution) through a sieve of 1mm mesh. For granulometric analyses the dried sediment was first sieved through a 1 mm mesh-size sieve. The mass percentage relative to the total sediment sample was determined for the sediment fractions larger than 1 mm. The sediment samples smaller than 1 mm were further analysed using a LS Coulter particle size analyser and sediment fractions were expressed as volume percentage of the 0-1 mm fraction. Median grain-size was determined based on the 0-1 mm fraction.

Five pieces of holothuroid origin were found and examined. These are now held in the collections of the Royal Belgian Institute of Natural Sciences under code IG 30055.

Order Apodida Brandt, 1835
Family Synaptidae Burmeister, 1837
Subfamily Leptosynaptinae Smirnov, 1989
Genus *Leptosynapta* Verrill, 1867
Leptosynapta minuta Becher, 1906
Figs 1 A-C; Map 1

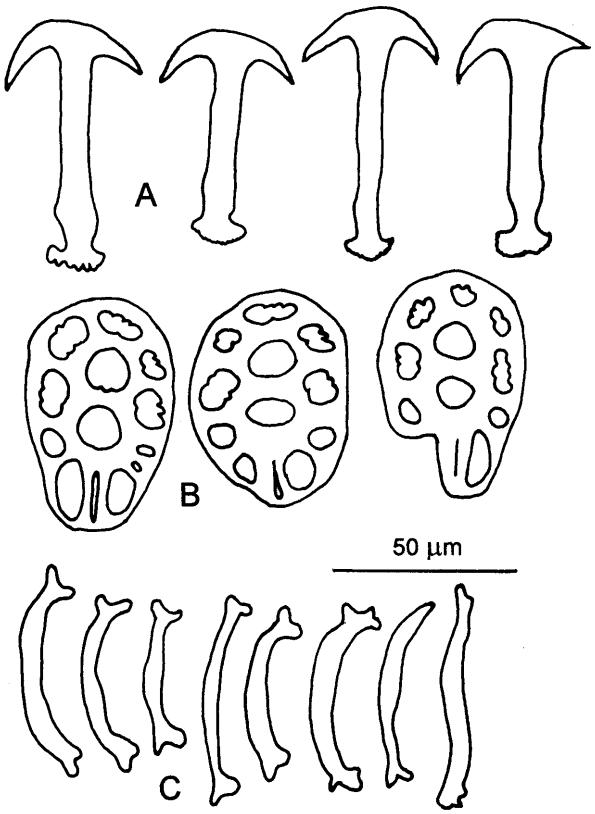


Fig. 1. – *Leptosynapta minuta* Becher, 1906. A : anchors from body wall; B : anchor plates from body wall; C : rods from tentacles.

Synapta minuta BECHER, 1906 : 507, text-Figs 1-3.

Leptosynapta minuta; H.L. CLARK, 1907 : 93; MORTENSEN, 1924 : 251; KOEHLER, 1924 : 272, pl. 15, Fig. 15; MORTENSEN, 1927 : 431, Fig. 262(4); MORTENSEN & LIEBERKIND, 1928 : 32, Fig. 62; HEDING, 1928 : 207; CHERBONNIER, 1951 : 13; CHERBONNIER, 1953 : 165, pl. 1, Figs A(a-l); CHERBONNIER, 1960 : 122, Figs 2(k-l); FIZE, 1960 : 505; MONNIOT, 1962 : 294, Figs 25(a-d); SWEDMARK, 1964 : 33; SWEDMARK, 1965 : 94; TORTONESE, 1965 : 103; CHERBONNIER & GUILLE, 1967 : 328; MONCHARMONT, 1968 : 513; SWEDMARK, 1971 : 45; SALVINI-PLAWEN, 1972 : 464, Figs 5 & 7; WILLIAMS, 1972 : 636; VAN DAMME & HEIP, 1976 : 44; TORTONESE, 1977 : 341; SALVINI-PLAWEN, 1977 : 75; TORTONESE, 1980 : 146; O'CONNOR, 1981 : 248; BESTEIRO & URGORRI, 1987 : 94; PÉREZ-RUZAFA et al., 1992 : 176; PICTON, 1993 : 84, textfig. (a); MADSEN & HANSEN, 1994 : 108, Figs 69-70, map 29; PANCUCCI, 1994 : 31; PANCUCCI, 1996 : 47, map 21; MCKENZIE, 1997 : 275; HANSSON, 2001 : 350; PICTON & MORROW, 2002 : http ://www.ulstermuseum.org.uk/marinelife/echinode/lepmin.htm.

1. *Morphology*

The five pieces represent at least three adult specimens (three pieces have a tentacle crown). Specimens are in poor condition, whitish grey in colour and with adhering sand grains. The largest of the pieces with a tentacle crown is 2.9 mm long and 0.9 mm across. It has ten digitiform tentacles without lateral digits. The two other specimens with a tentacle crown (6 and 4 visible tentacles) are respectively 2.4 mm long x 0.6 mm across and 2.2 mm long x 0.6 mm across. The two remaining pieces are both 1.1 mm long x 0.4 mm across. Due to the very small size of the specimens no observation of internal anatomy was possible without destroying them. Hence, the presence or absence of juveniles in the coelomic cavity could not be ascertained.

In the body wall there are anchors and anchor plates. Anchors (Fig. 1A) are 69.2 $\pm$ 3.4 µm x 37.5 $\pm$ 2.5 µm (n=12). The arms and vertex are smooth and the stock is finely dentate. The axis presents a small swelling close to the stock. The anchor plates (Fig. 1B) are oval, 62.3 $\pm$ 2.9 µm x 44.2 $\pm$ 2.8 µm (n=12), perforated by about 12 holes, and without a posterior bridge. The two central holes are generally smooth. The peripheral ones are smooth or serrated. If serrated they have between 1 and 5 very large teeth. There are three posterior holes, the central one very often reduced to a slit (Fig. 1B). Within the tentacles are small rods (Fig. 1C), 40-60 µm long, straight or slightly curved with forked extremities. Miliary granules are absent in body wall and tentacles.

2. *Habitat characteristic*

Coarse sandy sediment, with a median grain-size of 569 µm, characterise the sampling station. The dominant sand fraction (62.5%) is the coarse fraction (500-850 µm), followed by the medium fraction (250-500 µm) with 33.4%. The gravel fraction (>2000 µm) takes up 39.91% of the total mass, while the very coarse sand fraction (1000-2000 µm) takes up 31.18%. The sample was taken at a depth of 14 meters.

3. *Distribution (map 1)*

Germany (Helgoland : type locality), the Netherlands, Belgium, France (Roscoff, Banyuls, Marseille, Sète), Ireland (Galway), UK (Menai Bridge), Spain (Galicia), Italy (Leghorn, Napoli), Greece (N Evvoikos),? Northwest Africa.

This is the first record of *Leptosynapta minuta* in Belgian waters. It brings not only a new species record to the Belgian fauna but also that of the genus *Leptosynapta,* subfamily Leptosynaptinae, family Synaptidae and the new order Apodida.

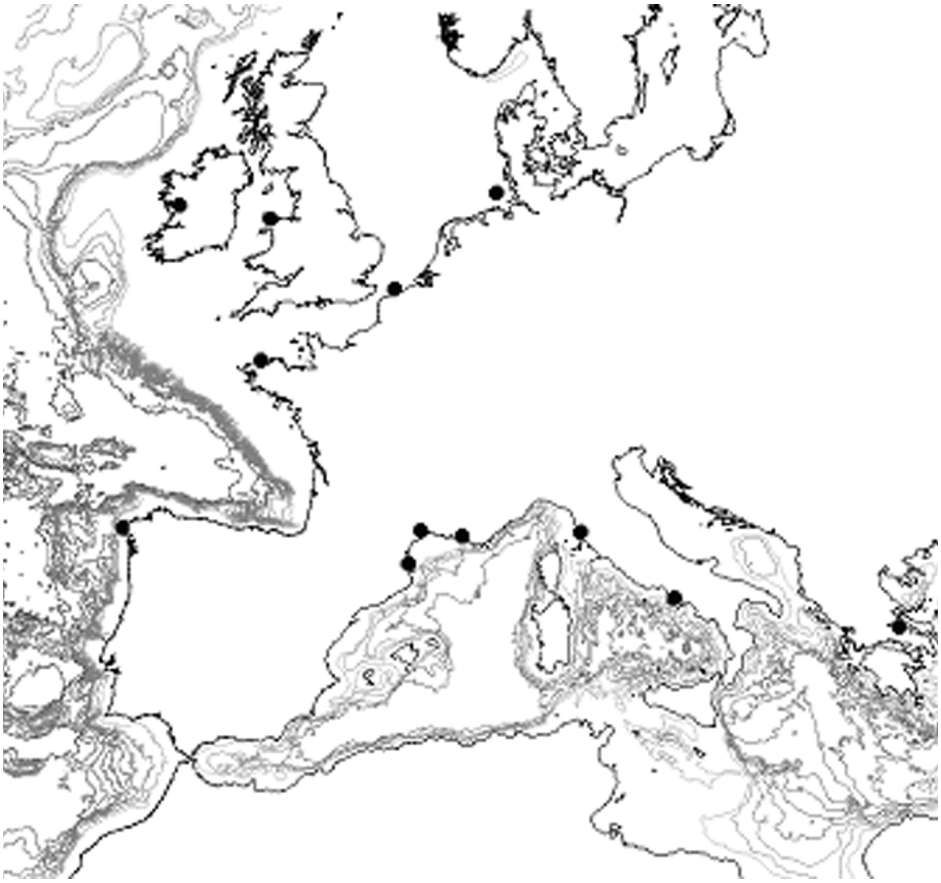
DISCUSSION

The very small size (body length less than 1 cm) of the specimens, their digitiform tentacles without lateral digits, and their small anchors (50-75 µm long) and anchor plates (60-68 µm long), are all characteristic of the species *Leptosynapta minuta* Becher, 1906. The shape and size of the ossicles are similar to that described by MORTENSEN (1927) for specimens from Helgoland, by CHERBONNIER (1953) for specimens from Roscoff, by O'CONNOR (1981) for specimens from Galway, and by CHERBONNIER (1960) for specimens from Banyuls. All the other species belonging to the genus *Leptosynapta* have larger ossicles : longer than 120 µm and very often reaching 200-250 µm (HEDING, 1928).

Leptosynapta minuta is found amongst maerl (marine sediment made of branched calcareous red algae) and coarse gravel in areas dominated by strong currents (PICTON, 1993; PICTON & MORROW, 2002). In addition the species is often associated with "*Amphioxus* sand", coarse sand present in the Mediterranean (FIZE, 1960; MONNIOT, 1962; CHERBONNIER & GUILLE, 1967) as well as along the European Atlantic coast (BESTEIRO & URGORRI, 1987). These habitat characteristics can also be found on the northern side of the Kwintebank (BONNE, 2003), the Belgian location. The depth at which the present specimens originated (14 m) is consistent with most of the data available in the literature : 2-20 m depth (MORTENSEN, 1927; FIZE, 1960; CHERBONNIER, 1960; CHERBONNIER & GUILLE, 1967; MONCHARMONT, 1968; SALVINI-PLAWEN, 1972, 1977; O'CONNOR, 1981). Only VAN DAMME & HEIP (1976) and PANCUCCI (1994) recorded specimens at a greater depth (35 and 60 m, respectively).

Leptosynapta minuta has an extensive distribution pattern (see map1) and is often regarded as a locally abundant species (CHERBONNIER & GUILLE, 1967; SWEDMARK, 1971). The locality "Northwest Africa", recorded by MADSEN & HANSEN (1994), could not be traced and is therefore dubious. It is surprising that *L. minuta*, a species with a reduced dispersal capacity (no planktonic stage due to brooding) has such a wide distribution.

It is surprising that *L. minuta* had not previously been reported from Belgian waters despite the huge sampling efforts over the last 30 years, both by macrobenthic (Van Veen grab) and meiobenthic (Reineck) sampling techniques. Probably, the post-sampling treatments used for macro- and meiobenthic samples caused the loss of *L. minuta* since, during sieving (alive) of macrobenthic samples, the species (width< 1mm) can easily pass through



Distribution of *Leptosynapta minuta* Becher, 1906.

the 1 mm sieve. Perhaps the species can only be found in samples which are fixed before sieving, a procedure not followed for samples taken in coarse sandy areas (Van Hoey et al., 2004). Meiobenthic samples, on the other hand, are treated with Ludox and centrifuged in order to separate the organisms and the sediment, as described in Giere (1993). Specimens of *L. minuta* are probably overlooked with such sampling techniques (except for a single specimen in the study of Van Damme & Heip, 1976) because they (1) cling to the sand grains or (2) have a different specific gravity compared to other meiobenthic taxa (e.g. Nematoda, Copepoda).

De Rycke (1982) and Denecker (1983) did report on the presence of holothuroid juveniles in Belgian marine waters but it was not possible to check whether these juveniles represented *L. minuta* as their material is no longer extant.

Data on the behaviour of *L. minuta* is scarce and somewhat contradictory. Everyone agrees that it is a brooding species, but opinions are divided regarding its position within the sand. According to Cherbonnier (1953 : 167) it does not crawl on the trivium as other synaptids but is positioned vertically in the sediment with the anus up. On the contrary, O'Connor (1981 : 248) reported "the animal lies on or within the sediment in a horizontal position. The calcareous ring is set in an oblique angle, allowing the tentacles to move over the substrate". Similarly, Picton (1993 : 84) mentioned that "the animal moves among the coarse particles in which it lives by crawling and wrapping its arms around the pieces of gravel or maerl". According to Swedmark (1964 : 33) the tentacles are strongly adhesive and help to move in the interstices.

These observations were most probably made using dredged specimens and not *in situ*. If this is the case it is not surprising to observe contradictory behaviours, which can be attributed to activities of disturbed animals.

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First record and morphometry of the non-indigenous fathead minnow *Pimephales promelas* (Rafinesque, 1820) (Teleostei, Cyprinidae) in Flanders (Belgium)

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KEY WORDS : *Pimephales promelas*, Flanders, first record, exotic species, morphometrics.

Pimephales promelas is widely distributed across North America. Its home area ranges from southern Chihuahua, Mexico, north to the Maritime Provinces and Great Slave Lake District of Mackenzie (Canada) and from the Rocky Mountains eastwards to the Appalachians. It has been introduced to both Atlantic and Pacific coastal drainage basins in the United States (1, 2). In Europe, reproducing populations of this non-native species have only been documented from the River Chiers in the Meuse basin in France (3).

The fathead minnow (*P. promelas*) is a robust, somewhat laterally compressed, cylindrical cyprinid with a maximal length of 100 mm. Its overall coloration is dark olive-green or brown above with a straw-coloured to whitish belly. At the base of the caudal fin, often a narrow dark vertical bar is present. A diagnostic feature is the shortened first ray in the dorsal fin. Males generally grow larger than females and have a typical swollen black head (4, 5, 6).

In 1995, the Institute for Forestry and Game Management (IBW) has made the first wild record of *Pimephales promelas* for Flanders (Belgium). Fish were collected using a pulsed-DC DEKA 7000 electrofishing unit with a ring anode. Three individuals were recorded in two small tributaries of the River Demer (Scheldt basin). Two specimens were captured in the Munsterbeek and a single specimen in the Zutendaalbeek. In 2001, ichthyological exploration of the Demer subbasin revealed 36 specimens of *P. promelas* from 7 localities in 3 different waters : Grote Gete, Melsterbeek and Cicindria (Fig. 1). The presence of *P. promelas* has never been recorded in other basins of Flanders, despite the numerous fish stock inventories by various research groups within the last 10 years.

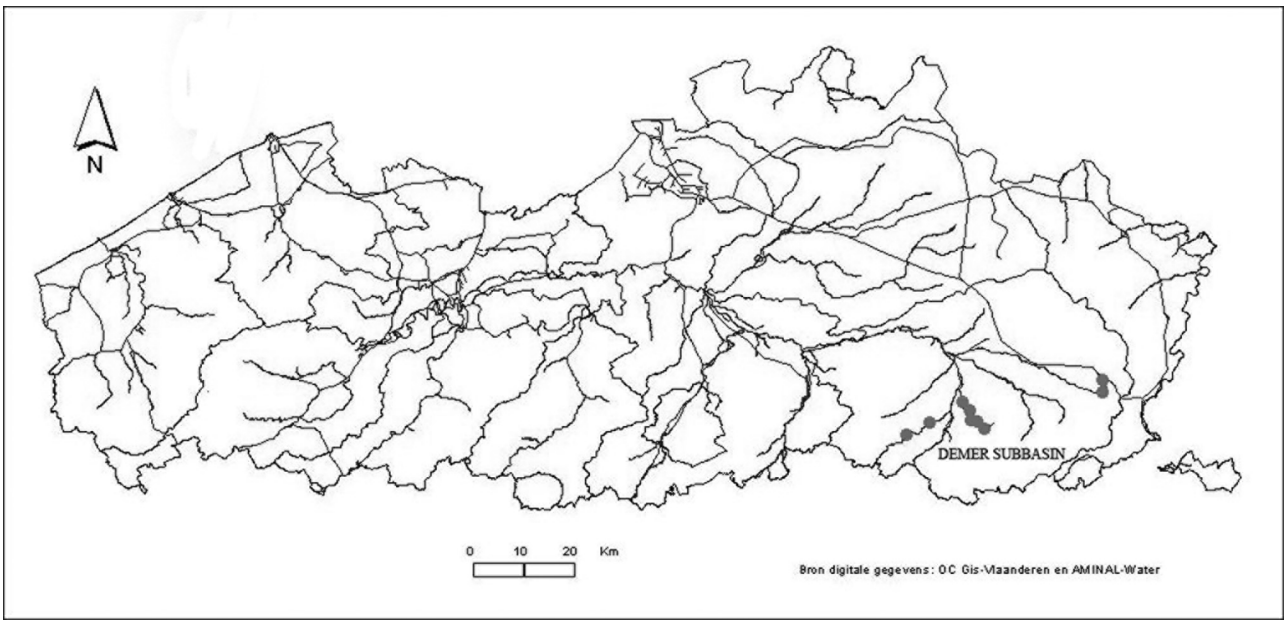

Fig. 1. – Map of the hydrogeographical network of Flanders (Belgium). The records of *Pimephales promelas* in the tributaries of the Demer subbasin are marked with spots.

A morphometric study was performed on different populations of this minnow species in North America (1). However, merely five morphological characters and three meristic variables were investigated. No data on the morphometry of introduced specimens of this alien fish in European countries are available by which a comparison can be made. We present here the results of a morphometric analysis exerted on 30 specimens from two different watercourses (Melsterbeek and Cicindria) of the Demer subbasin, which can serve as a base reference for future research. Thirty-seven morphometric variables were measured on the head and body of each fish specimen (Fig. 2). Measurements were taken point to point to 0.1 millimeter. The investigated specimens of *P. promelas* are housed in the fish collection of the Royal Museum for Central Africa in Tervuren (A1053-P-693-702 ; A1053-P-703-723).

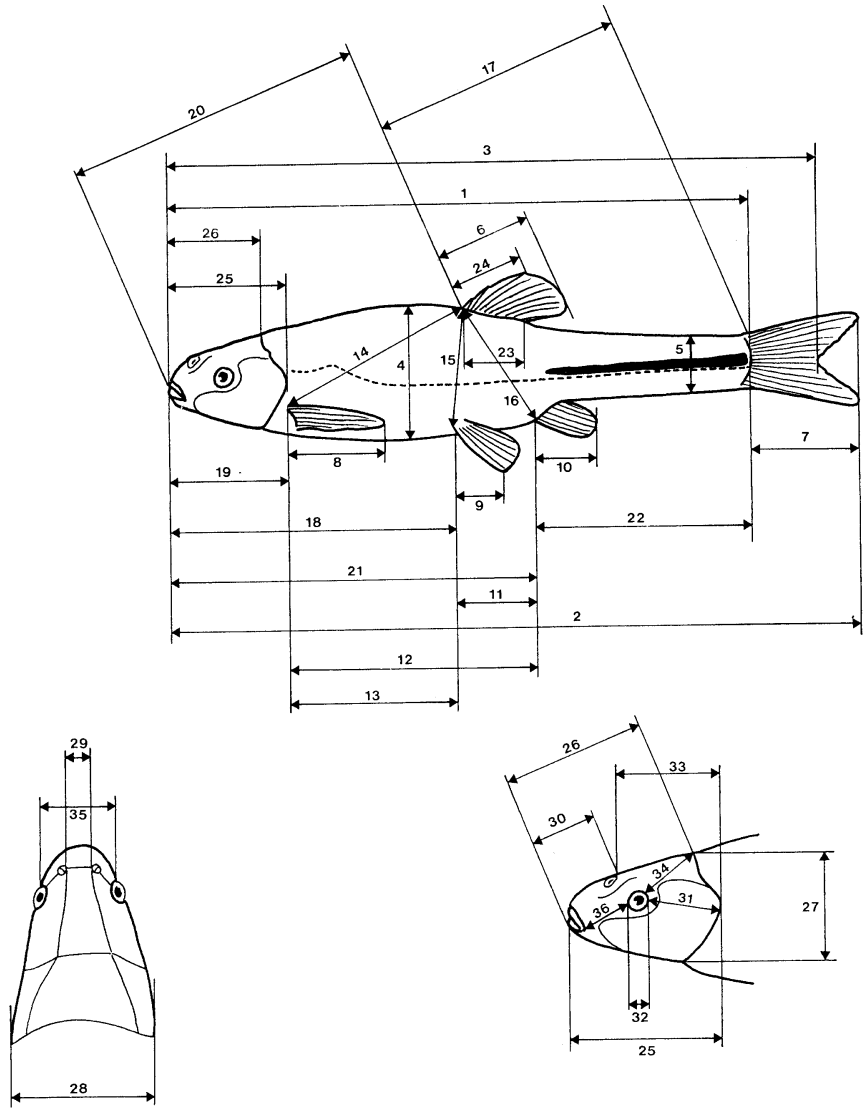

Fig. 2. - Location of the mensural measurements taken on the left side of the body and head and on the dorsal surface of the head of a *Pimephales promelas* specimen.

1 = standard length (ST), 2 = total length (TL), 3 = fork length (FL), 4 = maximum body depth (Mbd), 5 = minimum body depth (mbd), 6 = depth dorsal fin (DD), 7 = length caudal fin (LC), 8 = length pectoral fin (LPc), 9 = length ventral fin (LV), 10 = depth anal fin (DA), 11 = distance between anal fin and ventral fin (AV), 12 = distance between anal fin and pectoral fin (Apc), 13 = distance between ventral fin and pectoral fin (VPc), 14 = distance pectoral fin and dorsal fin (PcD), 15 = distance between ventral fin and dorsal fin (VD), 16 = distance between anal fin and dorsal fin (AD), 17 = distance between dorsal and caudal fin (DC), 18 = preventral length (PrV), 19 = prepectoral length (PrPc), 20 = predorsal length (PrD), 21 = pre-anal length (PrA), 22 = length caudal peduncle (LPC), 23 = length dorsal fin (LD), 24 = length of dorsal spine (Ldsp), 25 = maximum head length (MHL), 26 = frontal head length (frHL), 27 = head depth (HD), 28 = head width (HW), 29 = internasal length (ina), 30 = length snouth (LSn), 31 = postorbital length (Po), 32 = eye diameter (ed), 33 = distance between nose and operculum (nop), 34 = distance between eye and head-body transition (ehb), 35 = interorbital distance (io), 36 = distance between eye and mouth angle (ema).

TABEL 1-3

Morphological characters of male and female specimens of *Pimephales promelas* collected in two brooklets of the River Demer (Melsterbeek and Cicindria).

Melsterbeek n = 10 Character	Mean	Stdev	Min	Max	Var
ST	53,95	5,17	44,2	61,7	26,81
TL	66,13	5,52	56,08	75,12	30,48
FL	61,53	5,55	51,04	69,95	30,9
in % SL					
Mbd	23,93	2,03	20,18	27,01	4,13
mbd	11,71	0,93	9,49	13,03	0,87
Ldsp	14,94	0,88	13,21	16,17	0,77
LC	23,36	2,32	18,96	26,85	5,42
LPc	18,64	0,94	17,37	20,70	0,88
LV	15,02	1,06	13,58	16,92	1,14
DA	14,24	2,30	10,67	17,13	5,31
AV	16,24	1,15	14,98	18,63	1,32
VPc	27,88	1,59	24,50	29,79	2,54
APc	43,76	2,35	39,52	47,27	5,52
PcD	33,64	1,54	31,13	35,50	2,39
VD	22,19	1,85	18,28	24,98	3,44
AD	25,63	1,37	22,91	27,78	1,88
DC	49,55	1,42	47,84	52,05	2,03
PrV	52,30	1,33	50,08	54,05	1,79
PrPc	25,46	0,50	24,71	26,15	0,25
PrD	52,54	1,40	50,01	54,09	1,98
PrA	67,42	1,86	65,22	70,89	3,46
LCP	33,89	1,27	31,78	36,24	1,61
LD	12,92	0,84	11,65	14,17	0,70
LA	7,72	0,95	6,38	9,260	0,90
DD	22,25	1,38	20,11	23,96	1,90
MHL	24,45	1,02	22,34	25,92	1,04
in % MHL					
HW	60,81	3,29	57,62	67,50	10,84
HD	75,38	3,28	71,79	81,92	10,81
io	41,47	2,17	38,09	45,48	4,71
LSn	32,48	2,11	28,70	35,63	4,45
Po	49,52	2,70	43,31	52,04	7,30
ed	21,85	1,39	20,15	24,39	1,95
frHL	82,15	3,15	77,21	87,15	9,93
ema	22,55	2,28	20,35	26,54	5,22
ina	16,40	1,55	14,83	19,61	2,40
nop	75,75	2,18	72,19	79,34	4,76
lvl	23,41	1,09	21,65	25,22	1,19
ehb	41,84	1,59	38,38	43,46	2,55

Cicindria n = 10 male fish Character	Mean	Stdev	Min	Max	Var
ST	57,09	7,98	45,03	66,15	63,77
TL	68,18	8,64	55,60	77,85	74,67
FL	64,33	8,60	51,89	73,66	73,99
in % SL					
Mbd	26,57	2,00	23,45	29,55	4,00
mbd	12,63	0,57	11,75	13,61	0,32
Ldsp	13,65	0,93	12,57	15,01	0,87
LC	23,02	1,60	20,92	26,98	2,56
LPc	19,43	0,81	18,23	20,83	0,67
LV	16,08	1,00	14,67	17,48	1,00
DA	15,79	0,96	14,11	17,10	0,93
AV	15,56	1,18	13,39	17,32	1,39
VPc	44,44	3,15	38,55	48,21	9,92
APc	29,10	2,38	24,86	32,09	5,70
PcD	34,63	1,61	32,20	37,10	2,61
VD	24,48	1,68	22,40	26,98	2,85
AD	26,95	1,34	25,52	29,18	1,82
DC	49,56	1,17	47,62	51,43	1,38
PrV	53,63	1,83	50,82	55,43	3,37
PrPc	25,75	0,94	23,75	27,42	0,90
PrD	52,37	0,83	51,31	53,55	0,70
PrA	67,91	1,87	64,60	70,14	3,51
LCP	34,31	0,93	32,84	35,58	0,88
LD	13,64	0,39	12,80	14,08	0,15
LA	8,24	0,52	7,27	9,08	0,27
DD	23,17	0,78	21,96	24,07	0,60
MHL	25,28	0,81	23,96	26,63	0,67
in % MHL					
HW	65,24	4,14	60,44	73,58	17,14
HD	83,05	6,27	73,35	93,82	39,32
io	46,34	5,04	41,03	53,80	25,46
LSn	34,68	3,51	28,70	40,30	12,33
Po	50,12	1,76	47,14	52,89	3,10
ed	20,30	1,61	17,82	22,71	2,59
frHL	83,81	2,58	80,09	87,95	6,68
ema	24,11	3,79	19,12	29,85	14,41
ina	17,77	1,22	14,64	18,77	1,51
nop	75,97	2,77	71,73	80,84	7,72
lvl	25,83	2,27	21,31	28,81	5,18
ehb	44,59	3,18	39,47	49,71	10,16

Cicindria n = 10 female fish Character	Mean	Stdev	Min	Max	Var
ST	48,57	3,95	44,26	55,2	15,64
TL	59,63	4,55	55,35	68,22	20,72
FL	55,29	4,46	50,64	63,14	19,93
in % SL					
Mbd	27,45	1,18	25,19	29,01	1,41
mbd	11,69	0,62	10,46	12,67	0,38
Ldsp	14,10	1,28	10,84	15,31	1,64
LC	24,23	1,06	22,38	25,59	1,14
LPc	18,01	0,93	16,55	19,50	0,87
LV	14,33	0,86	13,20	15,47	0,74
DA	10,59	0,51	9,94	11,64	0,26
AV	17,32	0,97	15,58	18,58	0,94
VPc	47,83	2,34	44,68	51,87	5,47
APc	31,13	2,14	28,35	35,59	4,60
PcD	36,11	1,53	32,78	38,67	2,36
VD	26,08	1,49	22,84	27,59	2,23
AD	26,04	0,96	24,71	27,42	0,93
DC	50,35	1,01	48,61	52,08	1,03
PrV	53,49	1,37	51,78	56,45	1,88
PrPc	24,08	0,77	23,08	25,41	0,60
PrD	52,45	1,08	50,12	53,75	1,18
PrA	70,98	6,66	67,60	89,69	44,43
LCP	32,19	0,81	31,10	33,41	0,66
LD	13,06	0,72	11,94	14,14	0,53
LA	6,14	0,57	5,25	7,21	0,32
DD	20,22	1,85	16,03	22,61	3,45
MHL	24,08	0,60	23,17	24,89	0,36
in % MHL					
HW	64,79	2,93	59,90	68,53	8,64
HD	78,50	2,72	74,61	82,90	7,41
io	42,27	2,40	39,89	47,16	5,76
LSn	31,64	1,69	29,31	34,04	2,85
Po	47,65	1,21	45,64	49,16	1,46
ed	24,07	1,81	21,29	26,95	3,28
frHL	86,67	2,80	82,40	91,86	7,87
ema	20,18	2,00	16,85	23,97	4,02
ina	16,28	1,66	12,82	18,71	2,77
nop	75,81	2,37	71,87	78,22	5,61
lvl	24,75	1,44	23,26	27,91	2,09
ehb	44,29	2,11	41,79	47,46	4,47

Sexual dimorphism is particularly manifested by means of larger anal fin length and larger body size in male fish. All biometric data are summarised in Table 1-3. Yet, no wild-caught *P. promelas* juveniles have been reported from Flanders. As both genders were in breeding condition, and regarding its expanding distribution, the species seems to be established.

The fathead minnow has attracted much attention to man as forage fish, suitable for pond culture, and as bait-fish for trout angling (7, 8). In Belgium and adjacent countries, a xanthoric form of this species is being sold for ornamental purposes. Non-indigenous species have often been found to out-compete, prey upon, or bring diseases to economically or ecologically valuable native species (9, 10). With respect to the potential harm involved with the introduction of non-indigenous species and the possible invasivity of the fathead minnow, one should be aware of, and follow up the development of this non-native fish species.

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Seasonal variability of *Mytilopsis leucophaeata* larvae in the harbour of Antwerp : implications for ecologically and economically sound biofouling control

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KEY WORDS : *Mytilopsis leucophaeata*, biofouling control, ecology.

Mytilopsis leucophaeata Conrad, 1831, the Brackish Water Mussel, is a mytiliform bivalve (Mollusca, Bivalvia, Veneroida, Dreissenidae), which produces strong byssus to attach to hard substrates. *Mytilopsis leucophaeata* is a typical estuarine species, and thus resistant to a wide range of oligo- to mesohaline conditions (1). The species originates from the southern coast of the U.S. to Tampico, Mexico (2).

In 1835, it was first detected in Europe, in the harbour of Antwerp (3). After a period of apparent absence, *M. leucophaeata* is currently found along the coast of the North Sea from Germany into France and recently in Great Britain (4). Ballast water discharges from ships were identified as a major vector in the transfer of nuisance aquatic species, such as *M. leucophaeata*, from one area of the world to another. The fact that the species was not detected in Belgian waters over more than 50 years does not necessarily indicate the absence of *M. leucophaeata* along the European coast. Because of the morphological resemblance with the closely related *Dreissena polymorpha*, the Zebra Mussel, species-confusion may have arisen. When *M. leucophaeata* became an economic problem in the nineties as an important industrial fouler, attention was brought back to this relatively unknown species.

Any surface exposed to untreated water provides an opportunity for the settlement and subsequent growth of organisms. Because of the high temperature and the constant supply of food and oxygen, cooling water systems are an ideal habitat for *M. leucophaeata*. Given these perfect conditions, settlement occurs readily and growth can be rapid until it causes fouling at the heat exchangers and the tubes in the conduits and finally leads to the failure of the operational systems. This phenomenon is known as biofouling (5). Of all organisms causing fouling in cooling systems, mussels are known to cause the most serious problems (6).

The freshwater Zebra Mussel *D. polymorpha* causes major fouling problems in freshwater lakes and great rivers in the U.S.. Hence, the biology and possible control methods of the species are well examined throughout the years. Brackish water species, on the other hand, are far more resistant to environmental changes, which makes them particularly robust fouling species. The most effective and cheap control measure is the use of chlorination. It was only when the legislation on biocide draining became stricter (VLAREM II, 4.2.4., VLAREM II, annex 2.3.1.), that the magnitude of the biofouling problem by *M. leucophaeata* in the harbour of Antwerp became clear. In the near future, specific research on cooling water draining will be conducted and standard concentrations will be lowered. When the legislation on biocide draining in Belgium will get stricter, the use of merely chlorine will no longer be effective against biofouling. Other, (more expensive) methods have to be searched for to prevent fouling problems, caused by *M. leucophaeata*.

Adult mussels can shut their protective shell valves and stop byssus production to isolate their body from changes in the external environment (7), such as biocide-passage. The planctonic larvae and plantigrades are the most vulnerable life stages, and thus susceptible to the biocides. Hence, knowledge on the cyclic presence of *M. leucophaeata* larvae provides a basis for an ecologically and economically proper use of these detrimental chemicals (8).

The occurrence of D-shaped larvae of *M. leucophaeata* in relation to temperature (°C) and salinity (PSU) was investigated in the cooling system of BASF, Antwerp in the period 2000 – 2003. As such, the recruitment period(s) of *M. leucophaeata* were determined.

Three replicate quantitative plankton samples were taken by sieving 50 l water over a 63 µm mesh sieve. From 4 February 2000 until 20 December 2000, veliger densities were monitored on a weekly basis. From March 2001 on densities were monitored weekly from spring untill late autumn, but in wintertime, in absence of larvae, a biweekly monitoring interval was chosen. Environmental variables were monitored weekly all year long. Plankton samples were preserved in 70% ethanol and veliger abundance was expressed as number larvae per cubic meter.

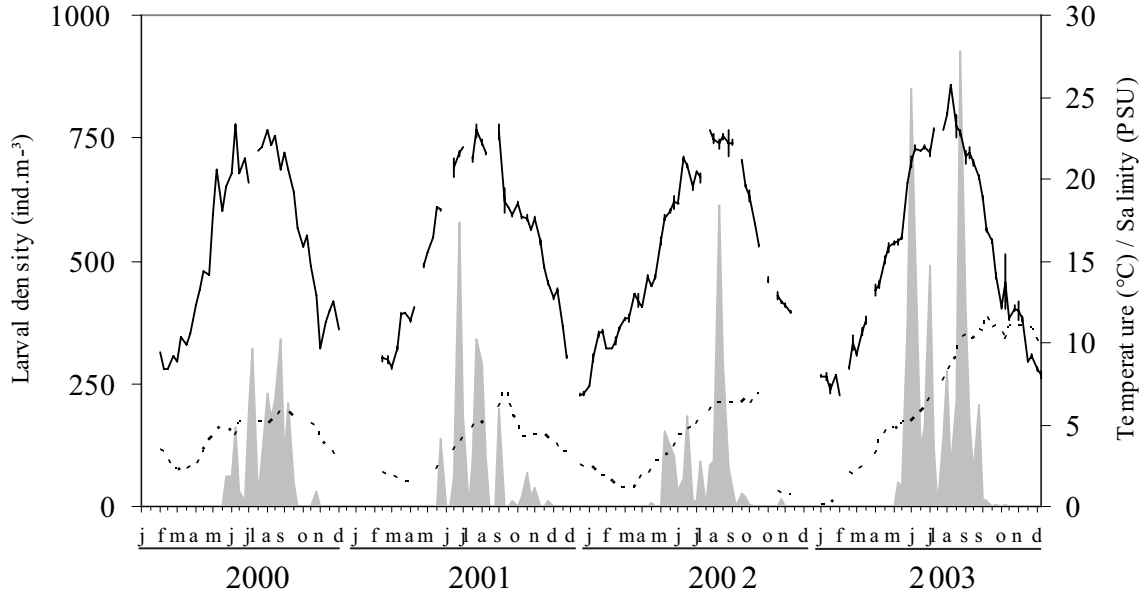

Fig. 1. – Seasonal variation in larval arrival of *M. leucophaeata* in the cooling water system at BASF, Antwerp (full line: temperature (°C), dashed line: salinity (PSU), grey shaded: larval density (ind./m³)).

Results indicated that in all years spawning began end of May – early June and lasted for about five months (Fig. 1). In 2000 larvae first appeared in the plankton at 3 June, in 2001 at 6 June, in 2002 at 21 May and in 2003 at 20 May. Temperature at first detection ranged between 16.2° C and 19.5° C, salinity ranged from 2.6 to 4.9 PSU. Annual and geographic variation in temperature has been identified as the primary factor triggering reproduction of *D. polymorpha* where veligers typically appear in the water at temperatures above 12° C. The intensity and duration of reproduction is believed to be controlled by an interaction of environmental factors (9), such as temperature, food availability and salinity. The variability in environmental factors indicates that for *M. leucophaeata* a threshold condition for spawning, like for *D. polymorpha*, does not seem to exist.

In all years, two or more distinct larval peaks could be observed. In 2000, 2002 and 2003 the highest peak occurred at the end of August-September (2000 : 340 ind./m³ at 7/9 ; 2002 : 613 ind./m³ at 20/8 ; 2003 : 927 ind./m³ at 26/8) at an average temperature of 21.4° C ± S.E. 0.4° C and salinity ranging from 5.1 PSU in 2000 to 10.3 PSU in 2003. In 2001, highest densities (580 ind./m³) were recorded earlier, at 3 July, when the water was 21.6° C and 3.9 PSU. After this peak, two smaller but distinct peaks were detected. The last peak occurred at 4 September and coincides with the highest peaks in the other years. Dreissenidae are sequential spawners, and the duration of larval production in *D. polymorpha* can vary from 6 to 52 weeks (10). The seasonal flexibility in larval production patterns indicates that adults carry ripe gametes for a very long time. After initial spawning, the exposure to ripe eggs and sperm in the water column often triggers gamete release by other ripe mussels, as such creating variability in recruitment.

In 2000, 2002 and 2003 larval densities declined after the highest peak and no veligers were found later then 19 November with average temperature 13 °C ± S.E. 0.4° C. Again, salinities were highly variable, ranging from 0.8 PSU in 2002 to 10.1 PSU in 2003. In 2001 last veliger densities were found on 20 November (13.7° C, 4.2 PSU). For *D. polymorpha,* 12 °C is the minimum temperature allowing gonad maturation and no veligers will appear in the water column at lower temperatures (11). Data show that for *M. leucophaeata*, this threshold temperature for gamete maturation may be 13° C ± S.E. 1° C.

The densities of larvae showed a high year-to-year variability, with moderate values in 2000-2002 (yearly average densities 2169 ind./m³ ± S.E. 78 ind./m³) and high values in 2003 (yearly densities 5273 ind./m³). This could indicate that the adult stock in the dock of BASF, Antwerp is still expanding. But although major differences in densities between months and years were found, the period of larval occurrence was markedly similar. The strict timing of larval presence of *M. leucophaeata* is a first indication that knowledge of the bivalve's life cycle can be an important tool in the combat against biofouling. To prevent new biofouling, a pointed dosage of biocides during the period of larval presence would be as effective as a continuous dosage throughout the year. A pointed dosage will decrease the amount of biocides needed, allowing to (1) meet the VLAREM II criteria on the use of biocides and (2) explore the use of ecologically less harmful, but more expensive biocides.

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A wingless intertidal ground beetle, new to the Belgian fauna, in the river IJzer estuary nature restoration site : *Bembidion nigropiceum Marsham, 1802*

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KEY WORDS : *Bembidion nigropiceum,* Coleoptera, Carabidae, intertidal beetle, first occurrence, expansion, river IJzer estuary.

At two occasions during the spring of 2003, we collected (by pitfall trapping) a brachypterous female of a small carabid beetle, *Bembidion (*subgenus *Lymnaeum) nigropiceum* Marsham, 1802, hitherto unknown from Belgium, in the river IJzer estuary at Nieuwpoort. The two sampling sites (2°43'58'' E – 51°09'00'' N; 2°44'05'' E – 51°08'56" N) are situated very near to each other within the area of a recent salt marsh nature development project (Fig. 1). The main digging and dike remodelling activities had been finished some months earlier only. A first specimen of *B. nigropiceum* was caught between 9 and 22 May, the second between 22 May and 6 June 2003. Both sampling sites are situated at the basis of a newly created artificial dike near the high tide water line, in an area with sand and some rubble. At both sites we regularly observed freshwater seeping out the dikes, but it is unclear whether the occurrence of the beetle is related to this phenomenon. During the same sampling periods, some 30 other sites were sampled with three pitfall traps each, within the context of a multidisciplinary monitoring project, along transects distributed over the entire study area, both in the newly created and already existing dune and salt marsh habitats. These sites have continuously been sampled for ground beetles for more than 15 years (9).

The geographic distribution of *B. nigropiceum* (Fig. 2, based on all data compiled from the literature, including unpublished data of Jeanne, in litt.) shows it is strictly confined to the tidal zone along parts of the Atlantic coasts, the Mediterranean and the Black Sea/Azov Sea. The closely related species *B. abeillei* Bedel, 1879 is known from other parts of the Mediterranean coast (France, Corsica and Spain), where it appears to replace its congener. A third species, *B. eichleri* Marggi & Wrase, 2002, has been recently described from the coast of Tunisia (2). In the UK, *B. nigropiceum* is confined to coastal localities (seven recent ones only) in southern England from Kent to Pembrokeshire, with a single recent record from south Wales (4), and is classified as nationally scarce (5). Because of its sporadic occurrence in the south it has been given special attention within the UK Biodiversity Action Plan. Little is know of the ecology of this beetle and it is probably constantly wingless, as opposed to the two other species of the subgenus *Lymnaeum*, which are winged. Until now, the beetle was also mentioned in Atlantic Europe from France only, from the Gulf of Morbihan northernmost up to Dieppe (north of the Seine estuary) (6, 7). In France, it has mainly been found under stones and in crevasses on rocky shores, which are submerged during high tide. In UK also, *B. nigropiceum* is found mainly in shingle and coarse sand, as well as among rubble at the base of cliffs, where it appears to have a partly interstitial way of life. The beetle has been found to 75 cm deep in the soil, especially where there is some detritus (8). It is supposed to have an annual life cycle, most probably breeding in spring with summer larvae (8). Both adults and larvae are supposed to be predatory on small invertebrates and may usually be subterranean. *B. nigropiceum* may be vulnerable to certain types of coastal development, but, on the other hand, can also temporarily appear on disturbed coastal sites between debris and stones in the intertidal (8). This could very well fit our own observations of the species in Belgium. Near Venice, a temporary but rather large population was observed in an artificial habitat near the harbour (8). The species probably disappeared again rather quickly because the habitat changed towards a much more fine-grained sandy beach. In that area also, the species lived at the basis of an artificial cliff protecting the narrow shore against sea-storms.

B. nigropiceum (Fig. 2) measures about 3,5 to 4 mm but does not at all look like a typical *Bembidion* species. Instead, it has a relatively large head with small and flattened eyes, a possible adaptation to an interstitial and partly subterranean way of life. It also lacks strong shoulders on the flattened elytra, possibly related to its constant brachyptery (as observed in other wingless beetles). Its coloration is unusual reddish-brown, as in other subterranean or cave ground beetle species.

The ecology, diversity and population dynamics of terrestrial invertebrates have been studied for many years along the river IJzer estuary, including the beachfront, coastal dunes and salt marshes. Ground beetles and spiders have been sampled and studied continuously since the early nineties. These long-term studies take place in the existing dune and salt marsh habitats and micro-habitats and even included sampling of available ecotones (gradients between habitats) and dikes (9). It is therefore very unlikely that even a small population of *B. nigropiceum* would have been overlooked earlier in this area. Whether a population of this beetle has been estab-

lished in this estuary remains uncertain, but will be monitored during forthcoming years. In view of its suggested habitat preference for coarse-sand to rocky shores this 'newcomer' might not be expected to be able to persist a long time in the area, in view of the fine-sand and silt soils that are expected in due time at these disturbed salt marsh restoration sites.

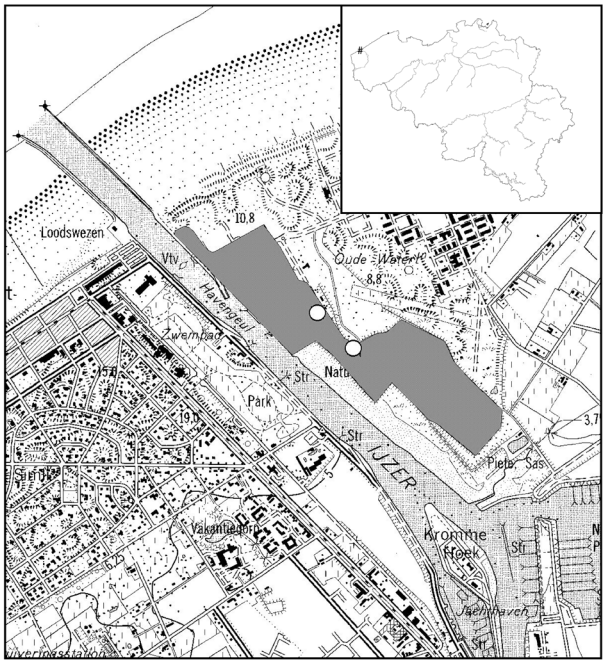

Fig. 1. – Detailed location of two sampling sites (white dots) in the river IJzer estuary where *Bembidion nigropiceum* was discovered in 2003; nature restoration area in grey.

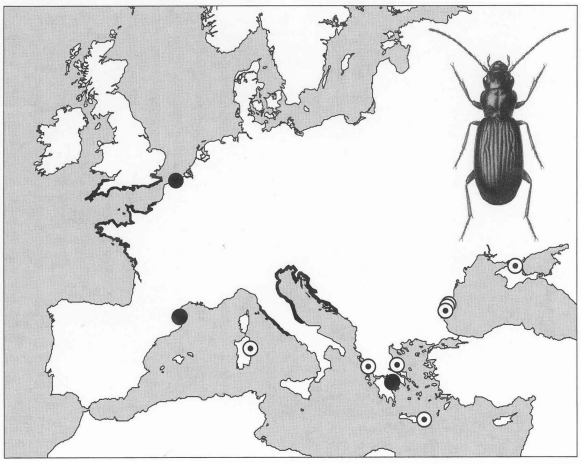

Fig. 2. – Geographic distribution of *Bembidion nigropiceum* (added figure; total length 4 mm) with more or less continuous areas in black, scattered sites (⊙) and new findings (●).

Possible sites of origin for these founding beetles only are known at large distance, both at the other side of the Channel (nearest sites with the species at about 100 km) and in France (at about 200 km). We cannot entirely exclude that the species might have been overlooked at somewhat closer sites, especially in the north of France. *B. nigropiceum* seems to be highly adapted to marine conditions, surely must support saline conditions and supposedly can survive longer periods of submersion by seawater. In view of its wingless state (brachyptery) and its special ecology, it is therefore hypothesised to have been washed ashore with floating debris originating from the south of England or the north of France. These recent captures add a new and peculiar ground beetle to the Belgian fauna and at the same time suggest that this carabid beetle is extending its range further north, a phenomenon recently observed for other terrestrial invertebrates in our region, e.g. the Mediterranean dwarf spider *Diplocephalus graecus* (10).

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Atypical mating behaviour in the empidine dance fly *Rhamphomyia* (*Lundstroemiella*) *magellensis* (Diptera : Empididae : Empidinae)

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KEY WORDS : Empididae, Empidinae, *Rhamphomyia* (*Lundstroemiella*) *magellensis*, mating and feeding behaviour.

Empidine dance flies are well known for exhibiting a large variety of mating and feeding behaviours especially mating swarms, transfer of nuptial gifts by males to females, production of silk cocoons as nuptial gifts, alternation between predation and flower visiting habit in correlation with mating and feeding periods respectively. These mating and feeding behaviours are well documented for a number of species belonging to various species groups (e.g. 1, 2, 3, 4, 5, 6, 7, 8), and now considered as autapomorphic for subfamily Empidinae (9). Some authors already suggested that this typical behaviour is absent and seems to have reversed in some species groups, such as the subgenera *Lissempis* Bezzi, 1909 (*Empis* L., 1758) and *Lundstroemiella* Frey, 1922 (*Rhamphomyia* Meigen, 1822) (10, 11, 12). However their suggestion was based only on morphological characters which seem to be incompatible with the typical behavioural traits (13); e.g. the presence of dichoptic eyes in the male suggests the absence of mating swarms. Detailed studies on such hypothesed reversed behaviour are not available.

Recently we succintly reported the atypical behaviour of a species of the subgenus *Lundstroemiella*, namely *R.* (*L.*) *magellensis* Frey, 1922 (14), for which swarming and nuptial gift are absent. The aim of this note is to give a complete description of the mating and feeding habits, including a detailed illustration of all the stages of the mating behaviour, as well as some hypotheses related to the evolution of the mating system of this species.

The behaviour of *R.* (*L.*) *magellensis* was observed for the first time in 1999 when one of us (C.D.) found the species in the French Pyrenees. Since the species was only known from mountainous regions of central Europe before (12, 15) and also because the endemism rate is rather high in Pyrenees (e.g. see 16), collected specimens were compared with specimens of the type series (Zoological Museum, Helsinki) to confirm their identity. Two successive field trips were subsequently organised in 2001 and 2002 in order to complete our previous observations. All field studies were performed in two different localities near the "Parc National des Pyrénées" (PNP), namely "Gavarnie, Plateau de Bellevue, limite du PNP, environ 1500 m" and "Gèdres, Granges de Bué, environ 1500 m" in June and July. The successive steps of the feeding and mating behaviour were photographed and videotaped with a digital video camera recorder. Pictures were then recovered and studied on a PC.

R. (*L.*) *magellensis* Frey is a typical mountain species of small size (3-4 mm), which is here recorded for the first time in the Pyrenees. Males and females are exclusively flower visitors, observed sucking nectar of *Valeriana* (Fig. 1A) and *Geranium* all day long. Many small flowers are to be found at the tip of each stalk of *Valeriana*, forming a rather large bearing surface on which individuals can easily move and meet. The mating behaviour as a whole takes place on this solid substrate and can be divided into three main successive stages.

In a first stage, when two males meet, and especially in the presence of a female close by, they often stop moving and face each other for impressing (Fig. 1B); a male can attack the other by jumping, this very quick fight generally ends with one of them taking flight. In this way, a male can stay on a same place trying to beat off competitors until a female comes or accepts the male for mating (Fig. 1C).

In a second stage, when the female allows the male to mount, competitors generally reappear quickly trying to evict and replace the mating male (Figs 1C, D). Consequently the competition between males is going on during mating and the female can support several males (Fig. 1D), moving from one flower to another to suck the nectar (Fig. 1E); this stage can last several tens of minutes.

In a third stage, the mating female leaves the dorsal surface of the plant to reach a sheltered place such as a blade of grass (Fig. 1F). At this place the male is generally no longer confronted to competitors and the mating pair is rarely disturbed by predators; the male grabs the thorax and abdomen of the female with its mid and hind legs respectively, whereas the fore legs are moved up and down (Figs 1G, H). Generally this stage lasts a very long time (until more than thirty minutes); mating always ends at an unpredictable moment.

From these field observations and in the light of the sexual selection theory (17), it is possible to formulate the following tentative hypotheses for the evolution of the mating system of *R.* (*L.*) *magellensis*.

There is a strong competition between males, including impressing and fighting, on the place where the individuals find their food resource, which therefore is also the place where they have a chance to meet females. We consider that this behaviour has something in common with the protection of a territory, and may be interpreted as a kind of selection ensuring females to get the best mates. During the first phase of mating, females continue to move on the surface of the flowers so that males are still in competition. It is hypothesised that this female behaviour plays the role of a second selection, although the male firstly selected is generally not evicted by competitors during this stage. Consequently females put males through a double selection : before and at the beginning of mating; after this selection stage and in all observed cases, they end up moving to a protected area where there are no longer potential competitors or predators.

There are three main differences in the mating behaviour of *Lundstroemiella* on the one hand and most of the remaining Empidinae on the other hand : first, the courtship behaviour takes place on a solid substrate in *Lundstroemiella*, not in swarms as it is usually observed in the subfamily. Second, the place where the courtship takes place is also there where males and females of *Lundstroemiella* find their food resource only consisting of nectar, whereas the remaining empidine species are generally flower visitors with males only becoming predator during the mating period. Third, there is no nuptial gift collected by males and offered to females in *Lundstroemiella* this protein-poor diet therefore implies uncommon physiological mechanisms for maturation of eggs (e.g. autogeny?) such as already hypothesised for other empidine dance flies for which silk cocoons used as nuptial gifts are empty or only contain a prey of very small size (18).

This descriptive work on the behaviour of *R.* (*L.*) *magellensis* should be considered as a preliminary and basic study for future investigations. However the mating behaviour of this species appears atypical within the Empidinae and, considering the rather derived phylogenetic position of *Lundstroemiella* within the subfamily (9), it is viewed as the result of a reversal from the traditional behaviour (mating swarms with nuptial gifts) usually observed. Such a mating behaviour is suspected for other empidine subgenera. Consequently, subject to discover this kind of behaviour in species belonging to these subgenera and to propose an extensive phylogeny of the empidine dance flies, such species groups, in addition to *Lundstroemiella*, could be relevant models to test evolutionary hypotheses such as the irreversibility of mating-systems evolution.

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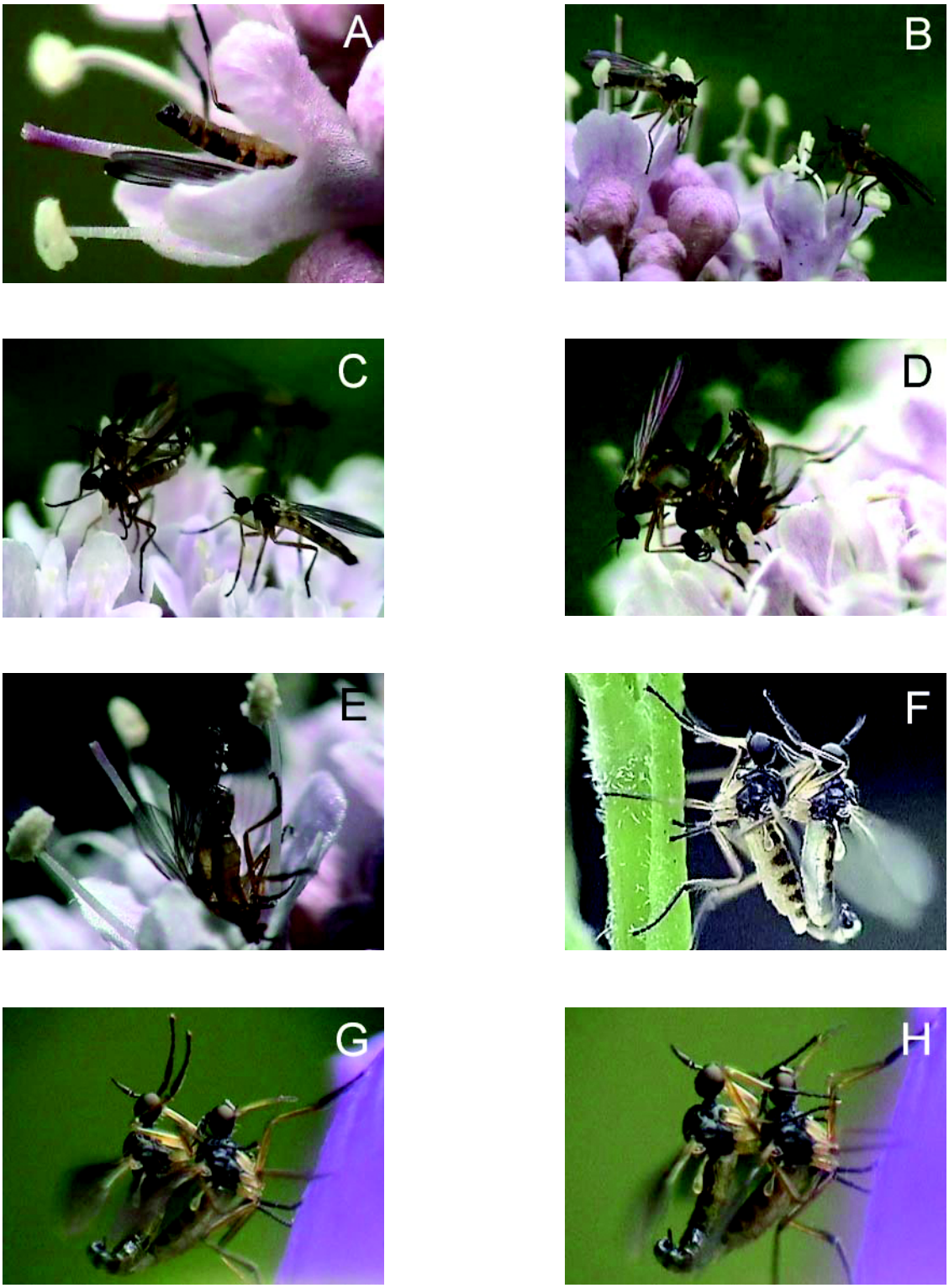

Fig. 1. – Sucessive stages of the mating and feeding behaviour of *R.* (*L.*) *magellensis*. A. – Male sucking the nectar of a flower of *Valeriana* sp. B. – Two males facing and impressing each other. C, D. – As soon as the mating is starting unpaired males reappear (C) and try to evict the mated male (D). E. – Although in copula, the female continues to move from flower to flower to suck the nectar during the first phase of mating. F, G, H. – Mating pairs on sheltered places during the second phase of mating.

Juvenile *Hippocampus guttulatus* from a neuston tow at the French-Belgian border

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KEY WORDS : Seahorse, neuston, floating debris.

The long-snouted seahorse *Hippocampus guttulatus* Cuvier, 1829 occurs mostly in shallow inshore waters among algae and eel grass (*Zostera* or *Posidonia*) and also in littoral lagoons (1). The species can be found in the Eastern Atlantic from the British Isles to Morocco, including the Canary Islands, Madeira and the Azores (2). The presence of the long-snouted seahorse in Belgian waters was already suspected (see CITES appendix II) but never confirmed. *Hippocampus ramulosus* Leach, 1814 is regarded as an invalid synonym of this species, although this name is still widely used for what is now *H. guttulatus* (2).

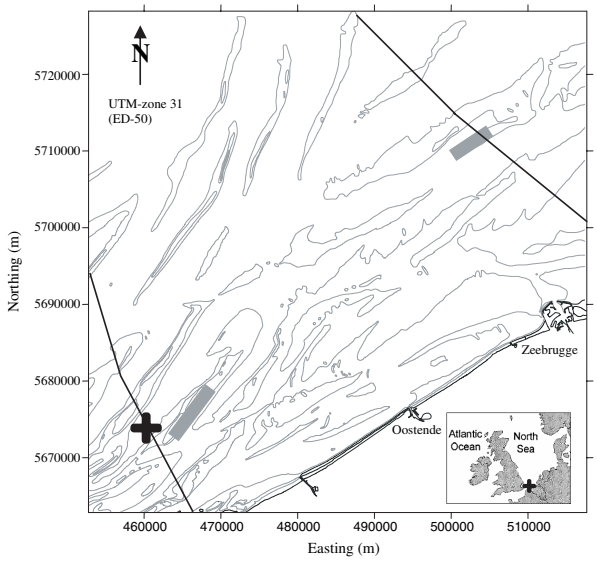

Fig. 1. – Sampling station *Hippocampus guttulatus* (black cross) and fishermen's catches of *Hippocampus hippocampus* (grey areas) in Belgian marine waters (border represented by black lines)

The sampling station where *Hippocampus guttulatus* was found is situated between the sandbanks Buiten Ratel en Oost Dyck (UTM 5674450 - 460236.7), on the French-Belgian border (Fig. 1). This station was investigated during a sampling campaign on August 20th (2003) with the research vessel 'Zeeleeuw'. The sample was taken with a rectangular neuston net (2m x 1m, 1mm mesh) from which only the lower 50cm is immersed, thereby sampling the upper 50cm of the water column. Identifications of the specimens, which were preserved in a 10% formaldehyde-seawater solution, are based on characteristics described by LOURIE et al. (2).

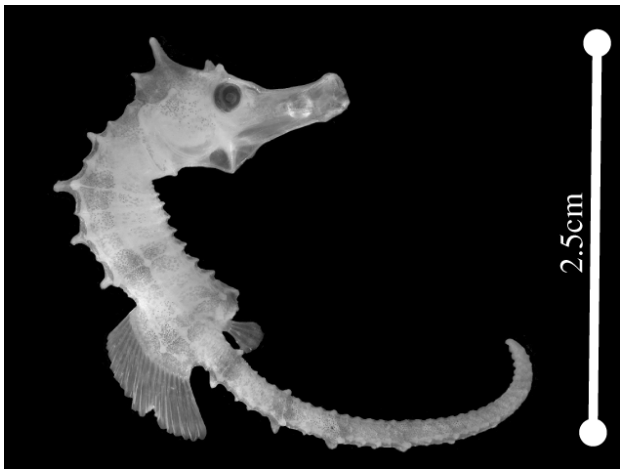

Fig. 2. – Photograph of specimen 2

The neuston sample contained two well-preserved juveniles (approximately 3 to 4 weeks old) of the species *Hippocampus guttulatus*. Most of the examined characteristics (Table 1) are within the range for both *Hippocampus guttulatus* and *H. hippocampus* (i.e. number of trunk rings, number of tail rings, number of pectoral fin rays, number of dorsal fin rays). However, the ratio of snout length to head length (> 1/3) certainly suggests that these specimens are *H. guttulatus* or the European long-snouted seahorse, and the number of pectoral fins on one of the specimens is consistent with it being *H. guttulatus*. Both specimens lack a mane of thick skin fronds on neck and head, usually seen in adults (Fig. 2).

TABLE 1

Examined identification characteristics for both specimens

	Specimen 1	Specimen 2
Overall height	2.93 cm	3.51 cm
Number of trunk rings	11	11
Number of tail rings	36	37
Snout length / head length	0.42	0.42
Coronet	Rounded knobs	
Spine development	Blunt and well-developed	
Pectoral fin rays	15	16
Dorsal fin rays	18	18
Cheek spine	Low and blunt	
Eye spine	Prominent, rounded	

TABLE 2

Recent seahorse catches (*Hippocampus hippocampus*) by local fishermen in Belgian marine waters

Year	Date	Number	Method of capture / vessel	Capture site	Destination of sea horses	Remarks
1997	May	4	Coastal fisheries	–	Oostende North Sea Aquarium	alive
1998	14-20 March	1	–	3 nautical miles from Zeebrugge	–	–
1998	24 July	1	Shrimp fisheries / O.211	-	Died and was discarded	During night
1998	22 September	7	Gill net / N.95	Between 51 12.70 N- 02 29.70E and 51 15.43N – 02 32.99E	released	Associated with *Alcyonidium*
1998	23 September	14			Aquarium of skipper-ship owner	
1998	24 September	33			Oostende North Sea Aquarium	
1998	25 September	66			Sealife Blankenberge	
1999	24 June	1	Coastal fisheries / O.101	–	–	–
1999	10 July	1	Coastal fisheries / O.152	–	–	Dead but no signs of decay
1999	14 July	1	O.20	–	–	Pregnant male
1999	–	–	N.95	Between 51 12.70 N- 02 29.70E and 51 15.43N – 02 32.99E	–	Skipper is convinced of the presence of a local population
2000	28 September	1	O.190	Westpit fishing grounds	–	Dead
2001	17 February	1	Gill net / O.369	–	–	–
2001	18 August	1	Coastal fisheries / O.190	–	–	–
2001	25 September	1	Coastal fisheries / O.190	–	–	Male
2002	11 September	1	O.191	Fishing grounds near Oostendebank	–	–
2004	20 April	1	O.190	Wenduinebank	–	–
2004	21 May	1	O.20	3 nautical miles from Oostende	–	Alive

This is the first reported catch of *Hippocampus guttulatus* for the Belgian waters, and the origin of the specimens is unclear. Although there are numerous records for southern Britain, it is unlikely that specimens could reach the Belgian coast due to their poor swimming ability and the lack of assisting currents (3). On the other hand, the presence of a local population seems unlikely because from 1997 onwards, only *Hippocampus hippocampus* was occasionally caught by local fishermen (Table 2, Fig. 1). As the specimens were caught near the sea surface, it is likely that they were carried to the Belgian coastal zone through the English Channel in association with floating debris (the sample contained decaying duckweed, plant seeds and pieces of plastic). However, the presence of seahorses (and their fry) in the neuston is quite uncommon. Only *Hippocampus erectus* has already been reported from the neuston in association with floating debris and vegetation (4, 5, 6).

As the entire genus *Hippocampus* is listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and both *H. guttulatus* and *H. hippocampus* are listed as Data Deficient by IUCN (World Conservation Union), it is most important to gather information on the presence and persistence of local populations of seahorse species to form the basis of legal protection and conservation. Hopefully, this note will be the starting point for a detailed record of Belgian seahorse catches.

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