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IN MEMORIAM

EMERITUS PROFESSOR DR. MAX POLL

(21 juillet 1908 - 14 mars 1991)

Max Poll (à droite) et Paul Brien vers 1970.

Max POLL est né à Ruisbroeck le 21 juillet 1908. Après avoir effectué ses humanités à l'athénée de St-Gilles, il entame des études de zoologie à l'ULB qu'il termine comme docteur en Sciences zoologiques en 1931.

Il débute sa carrière de zoologiste comme assistant chez Auguste LAMEERE puis chez Paul BRIEN où il se spécialise en entomologie par des recherches sur les organes excréteurs des Coléoptères.

En 1938 une possibilité lui est offerte au Musée Royal d'Afrique Centrale à Tervuren où il entre comme Attaché et gravit progressivement les échelons de la hiérarchie pour devenir Conservateur en 1948 puis chef du Département des Vertébrés. Ces nouvelles fonctions à Tervuren ont réorienté sa carrière vers l'étude des Poissons. Max Poll devient un spécialiste mondialement réputé de la faune ichthyologi-

que du Continent africain au Sud du Sahara. Sa contribution à la connaissance des poissons du bassin du Congo-Zaïre est considérable. Son activité incessante au sein de cette institution a doté le musée de Tervuren d'une des plus riches collections de référence de poissons africains au monde grâce à son dynamisme et au réseau de correspondants qu'il avait réussi à créer sur ce Continent. Il a acquis une expérience personnelle du terrain en participant à plusieurs missions au Congo Belge entre 1946 et 1956. Une des ces missions fut réalisée avec Paul Brien, elle avait pour objet l'étude de la reproduction du Protoptère et la récolte du matériel pour une étude embryologiques de son développement.

Dans ses fonctions au Musée d'Afrique Centrale, Max Poll a également déployé une activité de vulgarisation scientifique tout à fait remarquable en réalisant des dioramas et une présentation muséographique des collections publiques. Cette activité mérite d'être soulignée car à cette époque (années 60), il était le seul, dans les musées d'Histoire Naturelle de Bruxelles et de Tervuren, à se préoccuper de l'aménagement des salles publiques, activité considérée avec dédain par ses collègues. Peut-être a-t-il été inspiré sur cette voie par la mission taxidermique qu'il fit en 1956 pour collecter les dépouilles de mammifères spectaculaires (girafe, éléphant, okapi, buffle, etc.) destinées à être naturalisées pour décorer le pavillon du Congo Belge de l'exposition internationale de 1958.

La conscience qu'il avait d'être au service de la collectivité peut être aussi évoquée à propos du travail systématique, avant tout utilitaire, qu'il consacra aux genres et aux familles de poissons africains. Cet ouvrage abondamment illustré était un guide d'identification essentiel pour tout zoologiste œuvrant sur le terrain. Au moment de sa disparition il avait remis cette œuvre sur le métier et préparait une réédition complétée et actualisée. Son souci de mettre ses compétences au service de la collectivité s'est aussi matérialisé par une publication consacrée aux poissons marins de Belgique édité en 1947. Cet ouvrage est toujours consulté avec fruit par les zoologistes.

Son œuvre scientifique est considérable, la contribution à l'étude systématique et biogéographique des poissons du bassin du Congo est tout à fait fondamentale.

En 1954 il est rappelé vers l'Université, sollicité par Paul Brien pour enseigner la Zoologie Systématique, la Géographie et l'Ecologie animales. D'abord Chargé de cours (1954-1956) il est promu Professeur extraordinaire en 1956 puis Professeur ordinaire en 1959. Il poursuivra simultanément ses activités au Musée de Tervuren et son enseignement à l'ULB, ce sera pour lui l'occasion de diriger de nombreux travaux d'étudiants sur l'étude des poissons.

Max Poll est promu à l'honorariat en 1978. Il continue cependant à fréquenter régulièrement son bureau à l'Université pour y poursuivre ses études sur les poissons africains. Sa disparition, survenue le 14 mars 1991, interrompt une activité scientifique qui s'était quelque peu ralentie après le décès, durement éprouvé, de son épouse.

Ses mérites scientifiques lui ont valu des distinctions académiques : Membre de l'Académie Royale des Sciences Coloniales (1958), du Comité Directeur de l'IRSAC (1960) et de la Section des Sciences Naturelles de l'Académie Royale de Belgique

(1963). Il est en outre nommé Commandeur de l'Ordre de la Couronne en 1965. Il fut également Président de la Société Royale Zoologique de Belgique (1951-1952), ainsi que Président d'honneur.

Max Poll laissera le souvenir d'un homme bon, généreux et intègre, passionné par son métier et qui avait le don de communiquer son enthousiasme.

Jean-Jacques VAN MOL.

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HOST PARASITE RELATIONSHIP IN HYDATIDOSIS : COMPARATIVE ANALYSIS OF HYDATID CYST FLUID AND SHEEP SERUM

by

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SUMMARY

Various biochemical and physiological characteristics of fresh sheep serum were estimated and compared with those of pooled hydatid cyst fluid (HCF), isolated from *Echinococcus granulosus* cysts of ovine origin. Concentrations of the following compounds were determined : total protein, total albumin, total lipids, triglycerides, cholesterol, high density, low density and very low density lipoproteins, glucose, bilirubin, creatinine, uric acid, urea, calcium, inorganic phosphate, iron, sodium and potassium. Total activities of the following enzymes were measured : glutaminate-oxaloacetate transaminase, glutaminate-pyruvate transaminase, L-gamma-glutamyl transferase, alkaline phosphatase and α-amylase. Osmolarity and pH were also determined and total proteins were subjected to acetate cellulose electrophoresis. It was shown that compounds that are related to the carbohydrate and lipid metabolism, as well as nitrogenous catabolism, were predominantly present in HCF when compared with sheep serum. In contrast, the HCF contained few proteins and showed a low activity of enzymes related to protein metabolism. HCF, although osmotically comparable to serum of a putative host, is characterized by high levels of sodium and potassium. Microscopically, ammonium urate, amorphous phosphate and calcium carbonate crystals are found. The results are discussed in view of cestode physiology and metabolical (in-)dependence of hydatid cysts.

Key words : Echinococcus granulosus, hydatid cyst fluid, analysis, proteins, enzymes, lipids, carbohydrates, bilirubin, creatinine, uric acid, urea, calcium, phosphate, iron, sodium, potassium.

INTRODUCTION

Cystic echinococcosis/hydatidosis is part of a zoonosis, caused by the tapeworm *Echinococcus granulosus* (BATSCH, 1786). The adult parasite lives in the intestine of dogs and other canids. Shed proglottids and eggs infect intermediate hosts like sheep, cattle, horses, grazing on pasture contaminated with faecal material of infected dogs. Man, who is a dead-end host, is infected through close contact with infected dogs. The ingested eggs hatch and the oncospheres pass through the intestinal mucosa ; they are transported to the liver (or other organs) where they develop into hydatid cysts. Some of these cause severe complications such as intrabiliary and intraperitoneal ruptures, whereby liberated protoscoleces will form new cysts. Also, due to the presence of foreign substances in the freed hydatid cyst fluid, severe anaphylactic shocks may occur. The estimated global incidence of hydatidosis in man is more than 100,000 per year. In endemic areas, the surgical rate is more than 10/million population/year. Notwithstanding current experimental trials of chemotherapy (ECKERT, 1986), surgical treatment still remains the inevitable way of intervention. In the course of this type of treatment many technical difficulties often occur, sometimes demanding drastic procedures such as liver transplantation (LANDA GARCIA, 1989). Furthermore, many treated patients suffer from recurrent infections of hydatidosis, so that there is still a need for efficient chemotherapy and vaccines. It is believed that comparative biochemical studies on *E. granulosus* and on host tissues may eventually result in the development of adequate chemotherapeutic measures (VESSAL *et al.*, 1972). In the present study, we look into some biochemical features of hydatid cyst fluid (HCF), isolated from infected sheep. Values are compared with those found in the serum of sheep. Results are discussed in view of the physiological relationship between hydatid cysts and their hosts.

MATERIAL AND METHODS

A pooled sample (900 ml) of HCF, isolated from sheep was obtained through Leti Laboratorios, S.A. (Barcelona, Spain). The fluid was centrifuged at 4,000 rpm for 10 min and the supernatant was used for further analyses. The pellet was microscopically examined for organic and inorganic crystals as well as for the presence of protoscoleces to assess fertility of the hydatid cysts. Sheep blood was obtained from healthy sheep, stored at 37° C for 1 h, and left overnight at 4° C. Subsequently the serum was collected after centrifuging the blood at 900 rpm for 10 min. HCF and sheep serum were analyzed on an Hitachi System 704 unless indicated otherwise.

Protein analysis

1) The Biuret method (WEICHSELBAUM, 1946) was applied to determine the total protein content, using the Boehringer Mannheim HiCo Total Protein test. Additionally, an ATOM cellogel electrophoresis system was used to separate the

proteins on cellulose-acetate strips. For sheep serum, 5 µl serum was applied. HCF was previously incubated with 5 % TCA (trichloroacetic acid) overnight at 4° C and then centrifuged at 4000 rpm/10 min. The pellet was resuspended in 100 µl bidistilled water, and 5 µl of this was applied for electrophoresis. The strips carrying the separated proteins were stained with amido black (10 min for serum, 30 min for HCF). Proteinograms were scanned with an ATOM DIGISCAN (atom-430/429) and the amount of protein in the different fractions was calculated with respect to the amount of total protein that was measured with the Biuret method.

2) The total amount of albumin was analyzed according to DOUMAS *et al.*, (1971). Source of reagents : Boehringer Mannheim Albumin test.

3) Glutaminate-oxaloacetate transaminase (GOT) and glutaminate-pyruvate transaminase (GPT) were analyzed, using a Boehringer Mannheim Twin GOT/GPT test [GOT : BERGMEYER *et al.* (1986a) ; GPT : BERGMEYER *et al.* (1986b)].

4) L-gamma-Glutamyl transferase (EC 2.3.2.2) was analyzed, using a Boehringer Mannheim HiCo gamma-GT New test, following the method of PERSIJN and VAN DER SILK (1976).

5) Alkaline phosphatase (EC 3.1.3.1) was analyzed according to the DEUTSCHEN GESELLSCHAFT FÜR KLINISCHE CHEMIE (1972). Source of reagents : Boehringer Mannheim HiCo Alcalic Phosphatase Opt. test.

6) α-Amylase was measured colorimetrically using the Boehringer Mannheim α-Amylase PNP test. The method was according to RAUSCHER *et al.* (1985).

Lipid analysis

1) The total lipid content was determined with a Merckotest Total Lipids following ZÖLLNER and KIRSCH (1962).

2) Triglycerides : analysis was done using a Boehringer Mannheim Twin TG/CHO test according to the method of KERSCHER *et al.*, (1985).

3) Cholesterol : analysis was done according to KERSCHER *et al.* (1985) using a Boehringer Mannheim Twin TG/CHO test.

4) Lipoproteins : VLDL (Very Low Density Lipoproteins), LDL (Low Density Lipoproteins) and HDL (High Density Lipoproteins) were calculated and measured as follows : VLDL in serum were estimated as TG/5 ; since HCF cannot be expected to be entirely comparable to vertebrate serum, care should be taken for the interpretation of VLDL values for HCF. HDL was measured manually using a Boehringer Mannheim HDL-Cholesterol test. The LDL-cholesterol was calculated as : LDL-Cho = Total Cho - [HDL-Cho + VLDL-Cho] (FRIEDEWALD *et al.*, 1972). Since calculation of LDL-cholesterol involves VLDL-cholesterol, equal care is required in the interpretation of the values found for HCF.

Glucose analysis

Total monomeric glucose was determined according to ZIEGENHORN *et al.* (1977), using a Boehringer Mannheim Twin BUN/GLU test.

Analysis of nitrogen-containing metabolites

1) Total bilirubin was measured following the DPD-method (with 2,5-dichlorphenyldiazoniumsalt) of WAHLEFELD *et al.*, (1972). Source of reagents : Boehringer Mannheim HiCo Bilirubin test.

2) Creatinine was measured using a modification of the Jaffé method, according to BARTELS *et al.* (1972). Reagents were from a Boehringer Mannheim Creatinine test.

3) Uric acid was measured according to TOWN *et al.* (1985). Source of reagents : Boehringer Mannheim HiCo Uric Acid PAP test.

4) Urea was enzymatically determined according to PRENCIPE *et al.* (1983). Source of reagents : Boehringer Mannheim Twin BUN/GLU test.

Physiological analysis

1) Total calcium was measured automatically as well as by flame photometry (Eppendorf). In the automatic analysis we used the method of RAY SARKAR and CHAUHAN (1967). Source of reagents : Boehringer Mannheim Calcium test.

2) Inorganic phosphate was measured according to HENRY (1974). Source of reagents : Boehringer Mannheim Inorganic Phosphate test.

3) Iron was measured using the method of SIEDEL *et al.* (1984). Source of reagents : Boehringer Mannheim Iron test.

4) Sodium in HCF was measured on by flame spectrometry (Eppendorf) ; in sheep serum it was done with the CORNING 614 Na^+/K^+ ANALYSER. Preliminary measurements for HCF on the CORNING 614 Na^+/K^+ ANALYSER showed values that impeded appropriate quantification using this technique for HCF, so that this was confined to sheep serum only.

5) Potassium in HCF was measured by flame spectrometry (Eppendorf) ; in sheep serum it was done with a CORNING 614 Na^+/K^+ ANALYSER. Analyzing HCF potassium using the latter technique showed the same problems as for sodium and likewise two different techniques were applied for hydatid cyst fluid and sheep serum.

6) Osmolarity was measured using the freezing point method. For reference a standard dilution curve with NaCl was used.

7) pH was determined with a Beckman M3500.

8) Crystals were examined under a microscope and determined, taking into account the pH of the fluid and the solubility of the crystals.

RESULTS

The results of most assays are shown in Table 1. Microscopical examination of the sediments of HCF revealed the presence of ammonium urate, amorphous phosphate and calcium carbonate crystals. The 900 ml of fluid yielded 1.5 ml of packed protoscoleces. The cellulose acetate gel electrophoreses of serum and HCF proteins are shown in Figs. 1. and 2. ; albumin predominates in the proteinogram of sheep serum, in which also many bands can be distinguished that probably repre-

TABLE 1

Summary of analyses of E. granulosus *hydatid cyst fluid (HCF) and sheep serum.*
ND = not done.

TEST	HCF	SHEEP SERUM	UNITS
TOTAL PROTEIN CONTENT	0.06	7.74	g/dl
ALBUMIN	0.03	3.18	g/dl
GOT/ASAT	6	79	U/l
GPT/ALAT	0	17	U/l
g-GT	2	34	U/l
ALK. PHOSPHATASE	30	253	U/l
α-AMYLASE	21	48	U/l
TOTAL LIPIDS	135	370	mg/dl
TRIGLYCERIDES	60	19	mg/dl
TOTAL CHOLESTEROL	3	73	mg/dl
VLDL	[12]	3.8	mg/dl
LDL	[0.5]	26.2	mg/dl
HDL	11	43	mg/dl
GLUCOSE	105	57	mg/dl
TOTAL BILIRUBIN	0.01	0.08	mg/dl
CREATININE	0.63	0.96	mg/dl
URIC ACID	0.41	0.11	mg/dl
UREA	41	36	mg/dl
CALCIUM (HS 704)	12.52	9.60	mg/dl
CALCIUM (flame photometry)	14	ND	mg/dl
INORGANIC PHOSPHATE	1.70	4.65	mg/dl
IRON	27	104	µg/dl
SODIUM (CORNING 614)	ND	111	mg/dl
SODIUM (flame photometry)	340	ND	mg/dl
POTASSIUM (CORNING 614)	ND	18.8	mg/dl
POTASSIUM (flame photometry)	67.2	ND	mg/dl
Osmolarity	258	ND	mOsmol/l
pH	8.12	ND	6

sent the alpha 1,2, beta -and gamma globulin fraction as they can be found in human serum. The mean ratio albumin/globulin based on 3 gel electrophoresis scannings was found to be 1.47. The sheep proteinogram shows striking differences with respect to that of HCF proteins : albumin and a band that probably represents gamma-globulins predominate in the latter, giving a mean albumin/globulin ratio of 0.59.

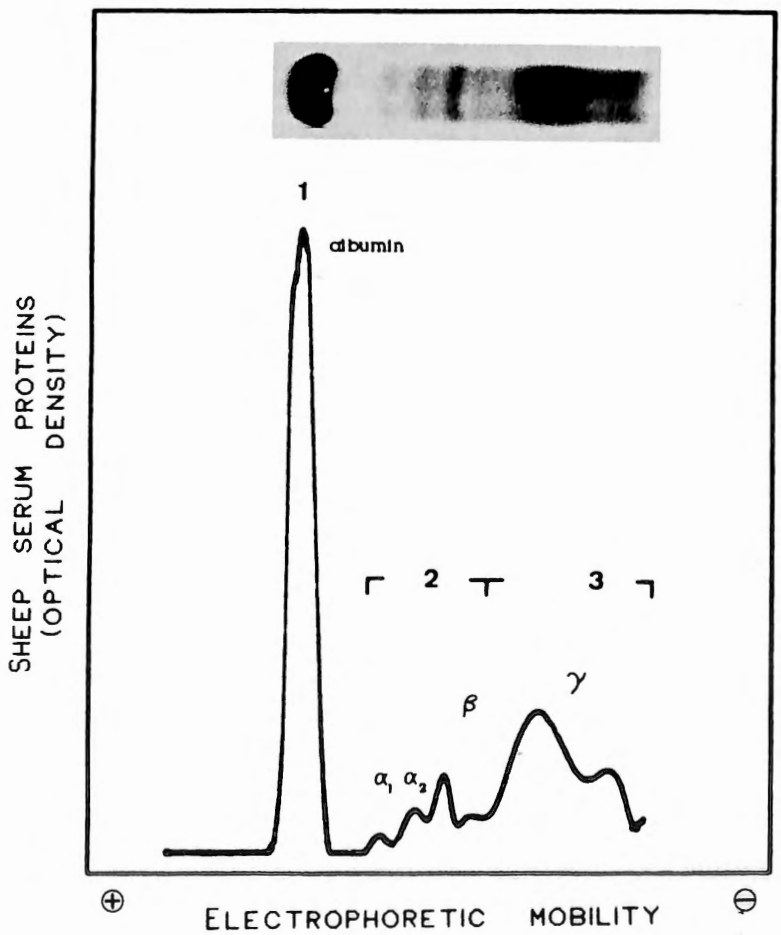

Fig. 1. — Cellulose acetate electrophoresis of sheep serum proteins. *Top :* stained gel ; *Bottom :* scanned gel. The amount of proteins per stained band (obtained after digitized scanning and considering the mean value of 3 gels) is calculated, based on a total protein content of 7.74 g/dl according to the Biuret method : b1 (albumine) : 4.60 g/dl ; b2.(alpha/beta globulines) : 0.50 g/dl ; b3.(gamma globulines) : 3.33 g/dl ; ratio albumin/globulin : 1.47.

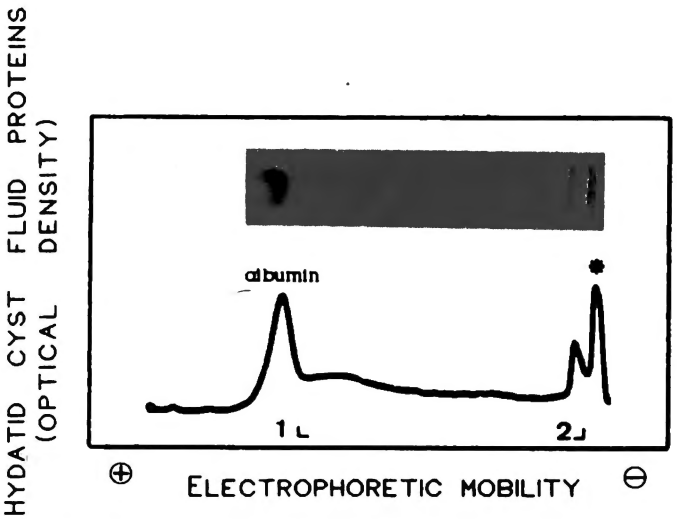

Fig. 2. — Cellulose acetate electrophoresis of HCF proteins. *Top :* stained gel ; *Bottom :* scanned gel. The amount of proteins per stained band (obtained after digitized scanning and considering the mean value of 3 gels) is calculated, based on a total protein content of 0.06 g/dl according to the Biuret method : b1.(alb) : 0.022 g/dl ; b2.(total globulines with a peak in the gamma globulin region) : 0.038 g/dl ; * = application situs of the sample ; ratio albumin/globulin : 0.59.

DISCUSSION

Protein metabolism

The HCF sample that we studied was found to contain 0.06 % (w/v) protein : this is about 130 times less than in sheep serum. Determinations of albumin and globulin indicated that these protein classes account for most of the total protein. Based on acetate cellulose electrophoresis, we found an albumin/globulin ratio of 0.59 in HCF and 1.47 in sheep serum. The striking differences in the distribution of the protein classes (see Figs. 1 and 2.), showing hardly any alpha or beta globulins, could be due to a selective accumulation mechanism in the cyst involving a higher rate of transport of the host immunoglobulins to the cyst tissue and fluid (cf. KASSIS and TANNER, 1977). How these proteins enter the cysts is not known but it may be by diffusion, through fissures in the cyst membranes (PAPPAS, 1978), or by endocytosis, which has been shown in the bladder of *Taenia crassiceps* (THREADGOLD and DUNN, 1983). In this respect, it is suggested that the ionic nature of proteins may also play an important role in their absorption (HUSTEAD and WILLIAMS, 1977). However, in view of the different distribution of predominant protein classes, and of the low amount of total protein found in HCF, it could be argued that the acquisition of host derived proteins is very low suggesting a signifi-

cant degree of biochemical independence on the part of the hydatid cyst residing inside an intermediate host. As an example of amino acid metabolism, we tested HCF and sheep serum for GOT, GPT and gamma-GT. gamma-GT is a cytosolic enzyme that acts within the so-called Meister cycle (the gamma-glutamyl cycle) that transports amino acids and several small peptides across the cell membrane. GOT and GPT are enzymes in the synthesis of amino acids (respectively L-aspartate and L-alanine). As such, GOT represents an important mechanism of transferring amino acids into useful compounds that can enter the Krebs cycle. Transaminations in general have been demonstrated in various cestodes (cf. SMYTH and MCMANUS, 1989). It is believed that, when compared to vertebrates, cestodes may have an extremely limited capacity for performing transaminations. This may reflect the fact that many cestodes live in an environment rich in amino acids, in which case synthesis may play a minor role in satisfying essential amino acid requirements. However, taking into account the very low amount of total proteins found in HCF, the parasitic fluid exhibited a considerable GOT activity (6 U/l). Sheep serum GOT on the other hand was much higher (79 U/l). No GPT activity was found in the HCF (17 U/l in sheep serum) and gamma-GT in HCF was 2 U/l (34 U/l in sheep serum). It should be noted that based on the ratio of enzymatic activity [sheep serum/HCF] (13.2 for GOT and 17 for gamma-GT) it cannot be decided whether these enzymes are host derived or represent metabolic activity of the parasite. The answer to this question could be positive as far as alkaline phosphatase activity in HCF is concerned : acid and alkaline phosphatases have already been demonstrated on the surface of protoscoleces of *E. granulosus* (MCMANUS and BARRETT, 1985). This finding supports previous evidence from in vitro studies that the scolex of *E. granulosus* can digest proteins at the host/parasite interface by membrane (contact) digestion (SMYTH, 1972).

Carbohydrates metabolism

α-Amylase is a hydrolytic enzyme that hydrolyses polymers of glucose containing α(1 → 4) glycosidic bonds (e.g. starch and glycogen). Several species of cestodes (incl. *Hymenolepis diminuta*) have been shown to absorb α-amylase, and adsorption appears to lead to an increase in amylolytic activity (BARRETT, 1981). In the case of adult cestodes this is a possible manifestation of the phenomenon of membrane (= contact) digestion. Our demonstration of α-amylase activity in the HCF could seem dubious : this particular enzymatic activity has hitherto never been detected in *E. granulosus* HCF. However due to the presence of glycogen in HCF (0.03 mg/ml) and in protoscoleces (218.76 mg/g lyophilized protoscoleces) (FRAYHA and HADDAD, 1980) this enzyme could provide an important mechanism for the parasite's carbohydrate metabolism. Earlier attempts to demonstrate α-amylase in larval *E. granulosus* have been unsuccessful (FRAYHA and HADDAD, 1980). To our knowledge this is the first demonstration of α-amylase activity in HCF. In conclusion : our results show that considerable quantities of sheep serum glucose could be taken up by hydatid cysts for consumption ; taking into account the earlier reports on the presence of endogenous glycogen, together with our demonstration

of α-amylase activity in HCF, our results also confirm the importance of carbohydrate metabolism in the biochemistry of *E. granulosus* hydatid cysts.

Nitrogenous compounds

Production of urea by cestodes in general suggests the existence of the urea cycle. One of the key enzymes, arginase, has been widely reported in cestodes (see SMYTH and MCMANUS, 1989). However, some of the other enzymes, notably carbamoyl phosphate synthetase and ornithine transcarbamoylase, are either absent, or present in only low quantities (BARRETT, 1981), and it is doubtful if a complete cycle operates in cestodes. It is likely that the urea excreted by tapeworms comes from the activity of arginase alone. We found the amount of urea in HCF to be 41 mg/dl. Since we determined the concentration of urea present in sheep serum to be 36 mg/dl it can be argued that the total urea present in the HCF is not entirely derived from host serum by diffusion or any other mechanism, but may be mainly of parasitic origin. In this respect it can be understood that HCF contains truly excretory products of protoscoleces, and the proliferative, germinal layer of the hydatid cyst which maintains a low urea concentration in the parasitic « tissue » could be a prophylactic measure to avoid deleterious effects on some vital processes like, for example, the inhibition of the phosphoenolpyruvate-pyruvate interconversion in the glycolytic scheme, (the blocking of this pathway by urea has been reported in certain ectoparasites (FRAYHA *et al.*, 1972). In our study we found 0.41 mg/dl uric acid in HCF, as compared to 0.11 mg/dl in sheep serum. These data confirm what has been said above in the case of urea, namely that HCF urea and uric acid represent proper parasitic catabolism. In addition, we refer to the abundant occurrence of ammonium urate crystals, present in the HCF. Measurements of bilirubin display a different pattern : the HCF contained 0.01 mg/dl and sheep serum 0.08 mg/dl. Since no data are available concerning the production or use of haemoglobin in cestodes, the present results suggest that the bilirubin found in HCF might indeed be host derived. In this respect bilirubin could represent a molecule of reference, due to its size, charge and relative quantity as compared to the host serum, to indicate which molecules present in the HCF could be parasitic and which derived from the host. In contrast, creatinine shows a ratio sheep serum/HCF of 1.52. With regard to the values found in HCF (0.63 mg/dl), creatinine has long been considered one of the end products of metabolism in *E. granulosus* (CODOUNIS and POLYDORIDES, 1936) despite the fact that FRAYHA and HADDAD (1980) found no creatinine in the protoscoleces. As is the case with other nitrogenous metabolites (such as urea), the pathways whereby these components are produced are almost unknown.

Lipid metabolism

Unfortunately, information on lipid-, phospholipid- and glycolipid metabolism in hydatid cysts is still scarce. Studies in this respect have been confined mainly to quantitative and qualitative examination of the lipid content and its distribution in

the larval stage of *E. granulosus*. Some data also exist on the total lipid content of *E. granulosus* adults and *E. multilocularis* protoscoleces. Recently, much of the available information has been comprehensively reviewed by FRAYHA and SMYTH (1983). Since it is generally accepted that lipid metabolism of cestodes is limited, it is likewise believed that these parasites largely depend on the acquisition of host lipids (*e.g.* for cholesterol see BAHR *et al.*, 1979). Our results, on the other hand, suggest that the lipid content of HCF is fundamentally different from that of sheep serum. The ratio [triglycerides/cholesterol] in the former is 20 whereas in sheep serum it was found to be 0.26. Furthermore, the measured ratio [total lipids/total proteins] is 2.25 in HCF and 0.05 in sheep serum. This suggests again the importance of lipid metabolism in the case of hydatid cysts and is evidence of their biochemical independence within the host. In addition it should be remembered that for instance the insect hormone ecdysone has been demonstrated in HCF (MERCER *et al.*, 1987). In the course of the present study we also compared lipids of different densities. Comparison of the ratio [high-density lipoproteins (HDL) : low-density lipoproteins (LDL)] between parasitic and host fluid reveals that HCF has considerably more HDL-bound lipids (22 as compared to 1.6 in sheep serum). This suggest that a relevant large portion of lipids in HCF is bound to proteins (about 50 % of vertebrate HDL weight consists of proteins).

Physiological parameters

Iron is required for the synthesis of the heme portion of hemoglobin and myoglobin. There are no data on the occurrence of hemoglobulin or myoglobulin in larval *E. granulosus*. However, the need for iron in cytochromes in the respiratory chain of the parasite suggests that a mechanism of iron absorption exists. In view of the relatively high amount of iron in HCF, this absorption (*e.g.* in the form of transferrin) could be a specific process. Sodium, potassium and chloride represent major ions of body fluids. Both sodium and potassium prove to be 3 to 3.5 times as concentrated in HCF, when compared with sheep serum. This indicates that these ions play an essential role in the physiology of larval *E. granulosus*. Calcium was found as $CaCO_3$ crystals in HCF. Soluble calcium was measured and showed to be higher in HCF than in sheep serum (ratio 1.3 to 1.4 depending on the method of assay). In view of the lower amount of inorganic phosphate in HCF as compared to sheep serum (ratio 0.36) it could be concluded that hydroxylapatite does not represent a major form of calcium storage in hydatid cysts. However, both ions are found as amorphous phosphate and calcium carbonate crystals in the HCF.

The finding of high levels of sodium and potassium in HCF raises the question of the osmotic and ionic relationships in hydatid cysts. We found the osmolarity of the HCF to be 258 mOsmol/l. Normal human serum has osmotic values of 285 to 295 mOsmol/l. Studies of water and electrolyte balance in protoscoleces of *E. granulosus* from sheep have been carried out *in vitro* by REISIN and ROTUNNO (1981) and REISIN *et al.*, (1981). Their data imply the existence of an active transport mechanism for Na^+ and K^+ and they suggest that the energy required to

maintain the Na-K balance within protoscoleces is largely provided by anaerobic pathways, with oxidative metabolism being only accessory to the energy balance.

CONCLUSION

A comparative biochemical examination of HCF and serum of a putative host suggests the following conclusion. In view of the relatively predominant amount of compounds related to carbohydrate and lipid metabolisms and nitrogenous catabolism, together with the low amount of total protein found in HCF, it is questioned whether much effort should be invested in the study of protein anabolism. Until now, attempts to produce commercially available vaccines based on larval *E. granulosus* material have proved unsuccessful. Recent strategies have been developed using genetic technology. However, we suggest more investigation of the carbohydrate and lipid metabolism of hydatid cysts, since in this respect too many gaps in our knowledge still exist. As mentioned above, ecdysone has been demonstrated in HCF. In addition, we are currently investigating HCF-derived lipid compounds with immunomodulatory activity. This is in line with the trend that treatment methods should be based on investigations of essentially different steps in parasite and host metabolism.

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FOURTEEN ROTIFER SPECIES NEW TO THE BELGIAN FAUNA, WITH NOMENCLATURAL AND TAXONOMICAL REMARKS ON SOME *SQUATINELLA* — SPECIES

by

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SUMMARY

Fourty-four rotifer species, of which fourteen are new to the Belgian fauna, were obtained from samples collected in the Botanical garden of the State University of Ghent (Belgium). Admittedly, of these fourteen species, seven are thermophilous and found only in a pool in a hothouse, but the remaining seven represent still a remarkably high number of additional species to the Belgian fauna. This can only be explained by the fact that most research efforts on Rotifera in Belgium were focussing on planktonic habitats. Nomenclatorial and taxonomical remarks on some *Squatinella*- species are added.

Key-words : Rotifera, distribution, *Squatinella.*

INTRODUCTION

During an International Training Course on Lake Management, which took place from October 1990 to March 1991 at the « Laboratorium voor Ecologie der Dieren, Zoögeografie en Natuurbehoud » of the University of Ghent (Belgium), zooplankton samples from ponds in the botanical garden of the university were studied. To our surprise, a considerable number of rotifer species not found in Belgium before, were identified. In the present contribution, the complete list of species, recorded from these samples, as well as remarks on some of the taxa present, are given.

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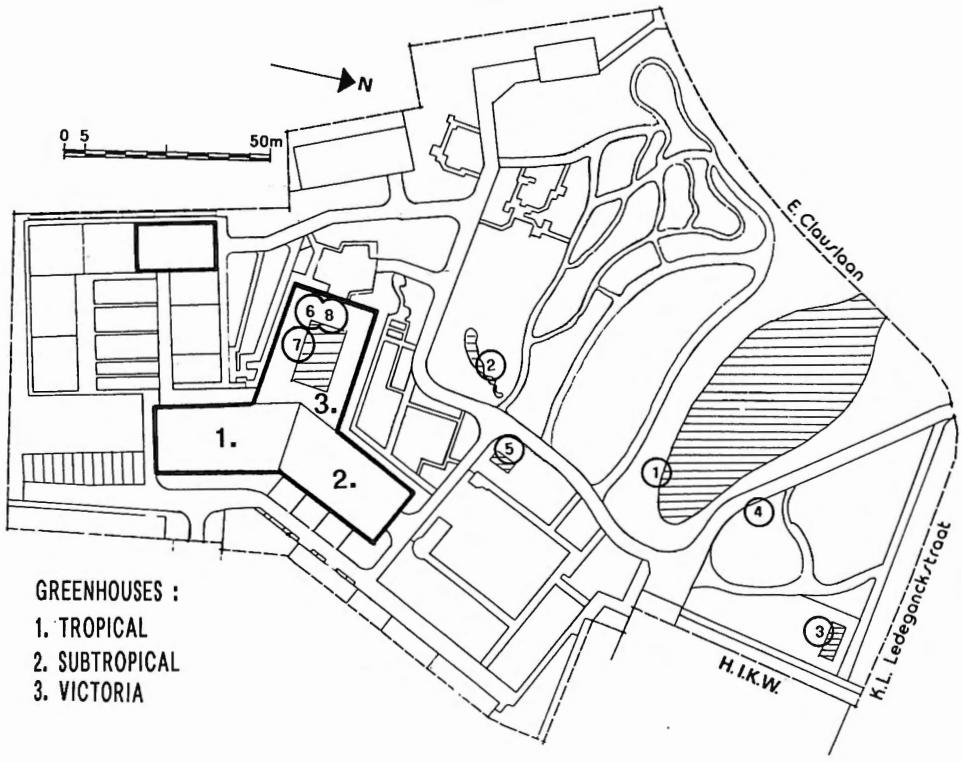


Map 1 : The botanical garden of the University of Ghent. Sampling points indicated by numbers as in table 1.

TABLE 1

Characteristics of the ponds in the botanical garden

- point 1 : large pond, vegetation dominated by *Hyppurus vulgaris,* water temperature : 8.7 °C.
- point 2 : middle of three connected ponds, running water. Scarce submerged vegetation of filamentous algae and mosses. Water temperature : 9.4 °C.
- point 3 : Concrete cistern, some submerged mosses and *Alisma plantago-aquatica.* Water temperature : 12.2 °C.
- point 4 : Stagnant water in small pit, with *Nymphar alba.* Water temperature : 9.8 °C.
- point 5 : Concrete cistern, vegetation of *Nuphar lutea.* Water temperature : 8.7 °C.
- points 6, 7, 8 : Large tank in hothouse. Samples 6 and 7 taken between vegetation, sample 8 : planktonic. Water temperature ranging from 23.2° to 31.0 °C.

MATERIAL AND METHODS

All the ponds studied are located in the botanical garden of Ghent university. A first series of samples was taken on 16 October 1990 in ponds 1 to 3 (see map 1). On 23 October 1990, a second series of samples was taken in these plus in five more sampling points. Three of these (6, 7 and 8) were situated in the pool of the « *Victoria* » hothouse. A photograph of one of the outside ponds (pond 2) was published by DUMONT (1987). Sampling was done with a hand plankton net (mesh size 50 µm). Some characters of the waters sampled are given in table 1.

RESULTS AND DISCUSSION

A list of the Monogonont rotifers found is given in table 2. In total, fourty-four species were observed, of which one *Cephalodella-* species could not be identified due to its rarity (only one specimen was found).

TABLE 2

List of the Rotifera, found in ponds in the botanical garden of the University Ghent.

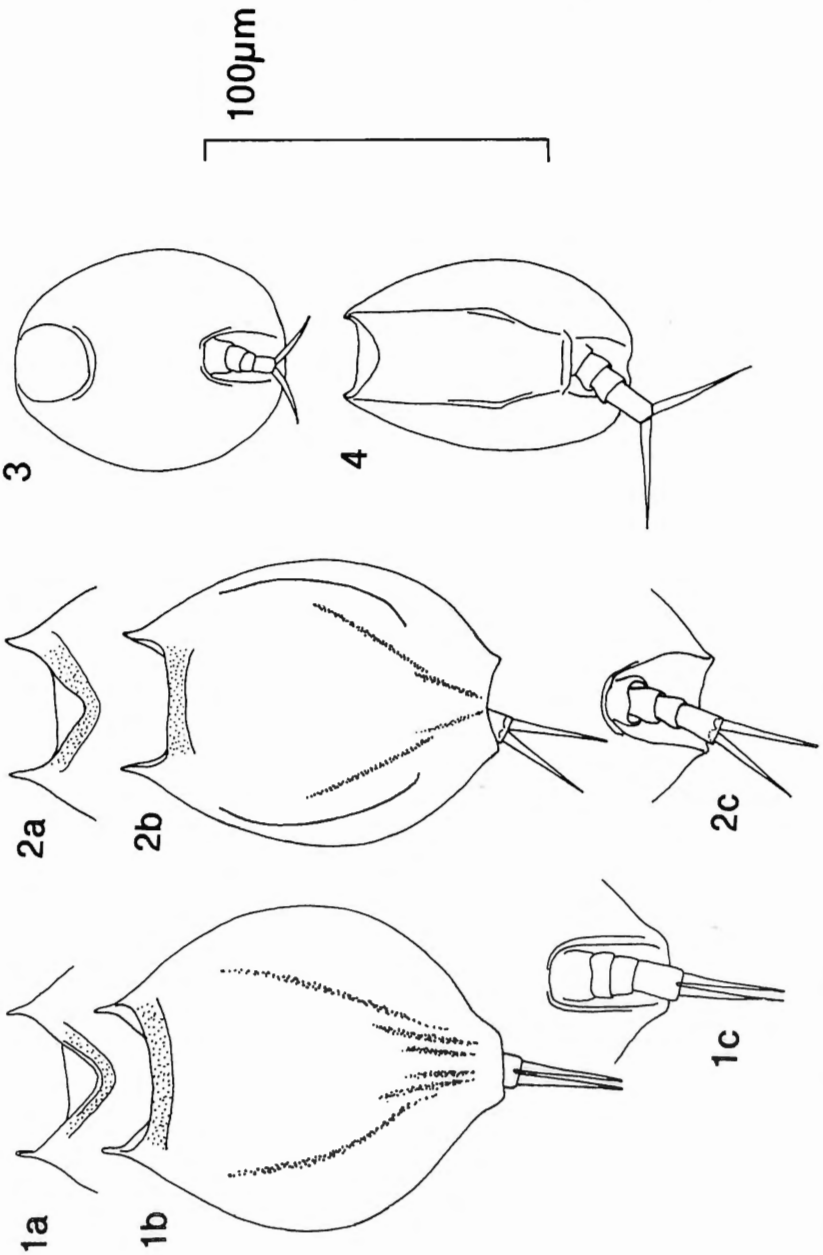
Species, marked with an asterisk are new to the Belgian fauna, crosses mark the presence of a species in the sample. Sampling was done on 16 October 1990 (pond 1 to 3) and on 23 October 1990 (1 to 8).

Pool :	1	2	3	4	5	6	7	8
Species :								
Brachionus rubens EHRENBERG, 1938			x					
Cephalodella forficula (EHRENBERG, 1932)							x	
Cephalodella gibba (EHRENBERG, 1932)			x					
Cephalodella ventripes (DIXON-NUTTALL, 1901)		x						
Cephalodella spec.			x					
Colurella adriatica EHRENBERG, 1931		x						
Colurella obtusa (GOSSE, 1886)							x	
Colurella uncinata (O.F. MÜLLER, 1773)							x	
Cupelopagis vorax (LEIDY, 1857)		x					x	x
Euchlanis dilatata EHRENBERG, 1832		x			x			
Keratella cochlearis (GOSSE, 1851)					x			
Keratella quadrata (O.F. MÜLLER, 1786)		x	x		x			
Lecane (*M.*) *bulla* (GOSSE, 1886)						x	x	
Lecane (*M.*) *closterocera* (SCHMARDA, 1859)	x	x	x		x		x	
Lecane (*M.*) *hamata* (STOKES, 1896)	x			x	x		x	

Pool :	1	2	3	4	5	6	7	8
Species :								
**Lecane (M.) pyriformis* (DADAY, 1905)	x	x	x				x	
Lecane (L.) flexilis (GOSSE, 1887)	x		x			x	x	
**Lecane (L.) glypta* (HARRING & MYERS, 1926)							x	
Lecane (L.) luna (O.F. MÜLLER, 1776)	x	x	x	x	x		x	
Lecane (L.) lunaris (EHRENBERG, 1832)			x					
**Lepadella apsida* HARRING, 1918							x	
**Lepadella costata* WULFERT, 1940							x	
Lepadella ovalis (O.F. MÜLLER, 1786)	x	x			x		x	
Lepadella paterlla (O.F. MÜLLER, 1786)	x	x	x	x	x		x	
**Lepadella quadricarinata* (STENROOS, 1898) var. *sexcarinata* KLEMENT, 1959				x				
**Lepadella triba* MYERS, 1934							x	
**Lepadella rhomboides* (GOSSE, 1868)	x			x			x	
Limnias melicerta WEISSE, 1848							x	
**Lindia torulosa* DUJARDIN, 1841								x
**Mytilina compressa* (GOSSE, 1851)		x						x
Mytilina mucronata (O.F. MÜLLER, 1773)	x	x		x				
Mytilina ventralis (EHRENBERG, 1832)	x	x	x				x	
**Notommata glyphura* WULFERT, 1935		x						
Platyias quadricornis (EHRENBERG, 1832)	x							
**Pleurotrocha petromyzon* EHRENBERG, 1830		x						
Polyarthra vulgaris CARLIN, 1943		x	x		x			
**Ptygura furcillata* (KELLICOT, 1889)							x	
**Sphyrias lofuana* (ROUSSELET, 1910)						x	x	x
**Spuatinella lamellaris* (O.F. MÜLLER, 1786)								
var. *mutica* (EHRENBERG, 1832)		x					x	
var. *tridentata* (FRESENIUS, 1858)		x						
Suatinella rostrum (SCHMARDA, 1846)		x	x	x			x	
Testudinella elliptica (EHRENBERG, 1834)			x	x				
Testudinella patina (HERMANN, 1783)		x						
Trichocerca cavia (GOSSE, 1886)		x						
Trichorerca rattus (O.F. MÜLLER, 1776)		x	x		x		x	

Of these fourty-four species, fourteen turned out to be new to the Belgian fauna. Seven of these occurred only in the hothouse. *Lepadella apsida* (Fig. 3), *L. costata* (Fig. 2), *Ptygura furcillata* and *Sphyrias lophuana* have a tropical or subtropical distribution or can, at least, be called thermophilous (KOSTE, 1978 ; SHARMA and SHARMA, 1987). It is likely that they represent accidental introductions. They may have been imported together with exotic plant specimens. The three other species (*Lecane glypta, Lepadella triba* (Fig. 4) and *Lindia torulosa*), as well as seven more species which were (also) caught outdoors (*Lecane pyriformis, Lepadella quadricarinata* var. *sexcarinata* (Fig. 1), *L. rhomboides, Mytilina compressa, Notommata glyphura, Pleurotrocha petromyzon, Squatinella lamellaris* in its varieties *tridentata*

(Fig. 5) and mutica (Fig. 6)) are all periphytic, living on and between submerged vegetation. Judging from the composition of a recent check-list of Belgian Rotifera (DE RIDDER, 1989), it seems that most research efforts in Belgium have been directed to planktonic habitats. It is therefore not surprising that a short but intense sampling campaign in the weedy environment of small water bodies yielded such



Figs. 1-4. *Lepadella quadricarinata* var. *sexcarinata* : Fig. 1, dorsal view (b.), ventral view of head aperture (a) and foot opening (c). *L. costata* : Fig. 2, dorsal view (b.), ventral view of head aperture (a) and food opening (c). *L. apsida* : Fig. 3, ventral view ; *L. triba* : Fig. 4, ventral view.

a remarkably high number of additional species. This view is supported by the capture of a periphytic species which is normally quite common (*L. pyriformis*) and whose absence in the list of Belgian Rotifera (DE RIDDER, 1989) can only be explained by insufficient sampling. The same holds true for *Pleurotrocha petromyzon,* but this species had in fact already been mentionned by BAIVERLIN (1984, unpublished dissertation) from the river Meuse.

One more taxon mentioned here is not listed in the review of DE RIDDER (1989). This is *Cephalodella ventripes* which has recently been recorded by DE SMET *et al.* (1988) from several ponds in the vicinity of Antwerp (DE SMET *et al.* 1988, 1989a, 1989b).

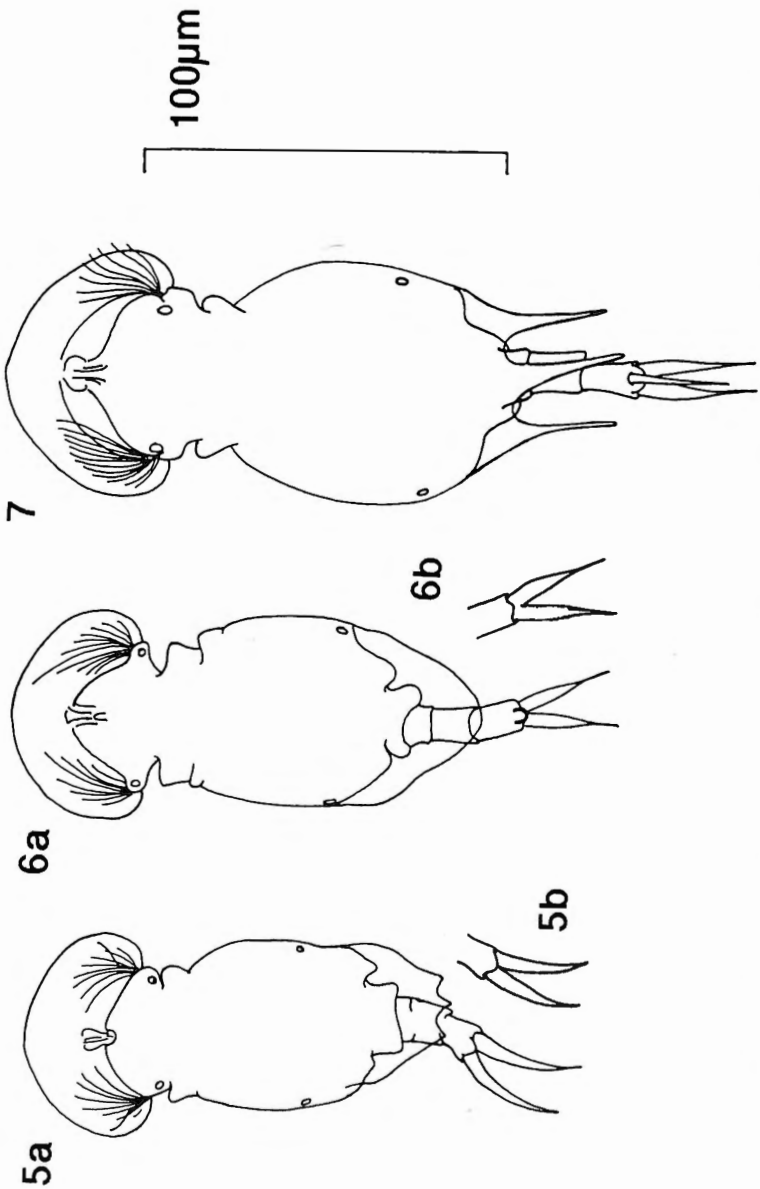
During our study, three morphologically different forms of *Squatinella* were found (Figs 5-7). They belong to a group of closely related taxa, characterised by the presence of a smooth dorsal lorica. From a taxonomical point of view, the three forms have been classified in several ways : KUTIKOVA (1970), PONTIN (1978) and BRAIONI and GELMINI (1983) believe that they represent different species, while PEJLER (1962) opines that all are synspecific.

A majority of authors, including VOIGT (1957) and KOSTE (1978, 1988) distinguish only two species, diagnosed by the presence or absence of a dorsal spine on the third foot segment (compare Figs 5, 6 with Fig. 7). The additional diagnostic characteristic used by KUTIKOVA (1970), PONTIN (1978) and BRAIONI and GELMINI (1983) is the presence (Fig. 5) or absence (Fig. 6) of three caudal extensions of the dorsal lorica. The diagnostic value of this characteristic is, however, low : a large number of intermediate forms have been recorded (CARLIN, 1939 ; KOSTE, 1978). It is therefore not maintained as a diagnostic characteristic for specific diagnosis.

PEJLER (1962) also rejects the validity of the first diagnostic characteristic, based on his observation of a specimen that seemed to be intermediate, and on WULFERT's description of *S. aurita,* in which a « spine » (dixit PEJLER, « Fortsatz » (= extension) by WULFERT, 1950) over the basis of the toes is reported to be occasionally present. The structure of the joint between the last foot segment and the toes (Figs 5-7) reveals that such an extension can indeed be present or absent, but its presence seems to result from a more pronounced retraction of the toes into the last foot segment and does therefore not represent intermediates between forms with or without a spine. PEJLER's (1962) observation must probably be interpreted in the same way.

As a result, only two taxa of specific rank are here recognised (apart from the insufficiently described *S. cirrata* (MÜLLER, 1773) : see KOSTE, 1978). In KOSTE (1978, 1988) the species provided with a spine is called *S. rostrum* (SCHMARDA, 1846) with as synonyms *S. lamellaris* (MÜLLER, 1786) (!) and *S. aurita* WULFERT, 1950. Evidently, this is erroneous as the name *S. lamellaris* is the senior synonym.

On the original figure of *S. lamellaris* by MÜLLER (1786, reproduced *in* VOIGT, 1957), no spine on the third foot segment is shown. Recognising this, CARLIN-NILSSON (1934) used the name to denote the taxon deprived of a dorsal spine on the third foot segment. HAUER (1936), however, argumented that it is possible that MÜLLER had overlooked this spine in his description, because of an observation by



Figs. 5-7. *Squatinella lamellaris* var. *tridentata* : Fig. 5, dorsal view (a), ventral view of foot and toes (b). *S. lamellaris* var. *mutica* : Fig. 6, dorsal view (a), ventral view of foot and toes ; *S. rostrum* : Fig. 7, dorsal view.

EHRENBERG (1838, *in* HAUER, 1936) who mentions but does not depict « eine Borste...dicht über den Fuszfingern » in his redescription of *S. lamellaris*. HAUER'S (1936) assumption can not be verified, but it has served as a basis for the synonymisation of *S. lamellaris* with *S. rostrum* by CARLIN (1939).

The alternative hypothesis that EHRENBERG's (1838) and MÜLLER's (1786) specimens, identified by these two authors as *S. lamellaris,* were not conspecific, is based on the observations provided by both authors and is favored by us. CARLIN's (1939) synonymisation of *S. lamellaris* with *S. rostrum,* based on EHRENBERG's observations, is therefore rejected. The synonymy of *S. aurita* with *S. rostrum* by KOSTE (1978) is also considered erroneous (see discussion above).
We conclude that the names *S. rostrum* and *S. lamellaris* (new synonymy : *S. aurita*), until proof of the contrary, are the valid names for the species with and without a spine on the last foot segment, respectively. In the highly variable species *S. lamellaris,* specimens with a differently formed caudal edge of the dorsal lorica occur. The nominate form has strongly developed caudal extensions of the dorsal lorica (as depicted by BRAIONI and GELMINI (1983) and WULFERT (1956)), but these extensions can be weakly developed (corresponding with *S. tridentata* FRESENIUS, 1858 : Fig. 5 ; in the combination *S. lamellaris* var. *tridentata*), or be absent (corresponding with *S. mutica* EHRENBERG, 1832 : Fig. 6 ; in the combination *S. lamellaris* var. *mutica*).

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FINE STRUCTURE OF THE GEMMA GLAND IN THE ANT *DIACAMMA AUSTRALE* (HYMENOPTERA, FORMICIDAE)

by

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SUMMARY

Workers of *Diacamma* have minute mesothoracic appendages termed « gemmae», and these represent a unique glandular structure among the Formicidae. In *D. australe,* each gemma contains approx. 500 glandular cells which are individually connected by duct cells opening through minute pores on the outer surface of the gemma. A specialized end apparatus forms the junction between the two cell types. Ultrastructural examination of the secretory cells shows the presence of both smooth and granular endoplasmic reticulum, and the occurrence of both electron-lucid and electron-dense inclusions. The secretion may therefore have a rather complex composition. Its function is presumably related to the peculiar mechanism of reproductive regulation found in this queenless ant.

Keywords : Diacamma, gemma, morphology, ultrastructure, exocrine glands.

INTRODUCTION

All species in the ant genus *Diacamma* (subfamily Ponerinae) lack queens. Instead, a single worker (= gamergate, PEETERS and CREWE, 1984) mates and reproduces in each colony. Another characteristic of *Diacamma* is that all workers emerge from the pupal cocoon with a pair of sac-like mesothoracic appendages. These tiny structures, which are homologous to the mesothoracic wings in queens (TULLOCH, 1934 ; BITSCH and PEETERS, 1991) do not exist in other ant genera, and have been called 'gemmae' (PEETERS and BILLEN, 1991). In each colony, only the gamergate retains the gemmae, because she aggressively removes them from every

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newly-eclosed worker (FUKUMOTO *et al.*, 1989 ; PEETERS and HIGASHI, 1989). Mutilation of the gemmae has important social consequences, since individual behaviour is affected, together with the ability to mate. We recently discovered that the gemmae in *Diacamma australe* (FABRICIUS, 1775) are completely filled with glandular cells (PEETERS and BILLEN, 1991). In the present contribution, we report on the fine structural organization of this novel gland in *D. australe*.

MATERIAL AND METHODS

The ants examined were obtained from a colony of *D. australe* excavated near Townsville, North Queensland (Australia). Gemmae of both callow workers and the gamergate were fixed in 2 % glutaraldehyde, buffered at pH 7.3 with 0.05 Na-cacodylate and 0.15 M saccharose. After postfixation in 2 % osmium tetroxide, tissues were dehydrated in acetone and embedded in Araldite. Thin sections were double stained with a LKB Ultrostainer, and examined with a Zeiss EM 900 electron microscope. Specimens for scanning electron microscopy were coated with gold-palladium and viewed with a Cambridge 360 microscope.

RESULTS

The gemmae appear as orange to brownish mesothoracic appendages in the region where wings normally occur in queens. They fit into a thoracic cavity, which can conveniently be designated as the 'gemmarium' (Pl. I). Each gemma has a length of approx. 450 µm and a width of approx. 200 µm. The anterior part forms a narrow stalk that articulates with the thorax, while the posterior part is densely packed with glandular cells (Pl. II, A and B). The outer (dorsal) cuticular lining measures about 10 µm in thickness, while the inner (ventral) lining hardly exceeds 2 µm (Pl. II, A). Approximately 500 glandular cells are estimated to occur per gemma, each being accompanied by a duct cell. Because of the dense concentration, the secretory cells assume a polygonal shape with a diameter ranging from 12 to 20 µm (Pl. II, B). Nuclei generally are very rounded with a diameter around 10 µm.

The end apparatus, which is the characteristic functional device for accumulation and release of secretion at the cellular level in this gland type, presumably is rather short because it is not always seen in a plane section (Pl. II, B). It consists of a cuticular collecting duct, surrounded by a sheath of microvilli. The cuticle shows a discontinuous epicuticular inner layer and a granular procuticle. The microvilli in general are more or less closely packed (Pl. III, A), although they may also appear in a much more disorderly arrangement because of considerable spaces distorting them (Pl. III, B). These differences may occur among the secretory cells of the same individual. Both electron-lucid and electron-dense spherical inclusions of variable diameter are dispersed in the cytoplasm (Pl. II, B ; Pl. III A, B). Other cytoplasmic elements include both smooth and granular endoplasmic reticulum (Pl. III, C), numerous mitochondria and randomly-scattered free ribosomes.

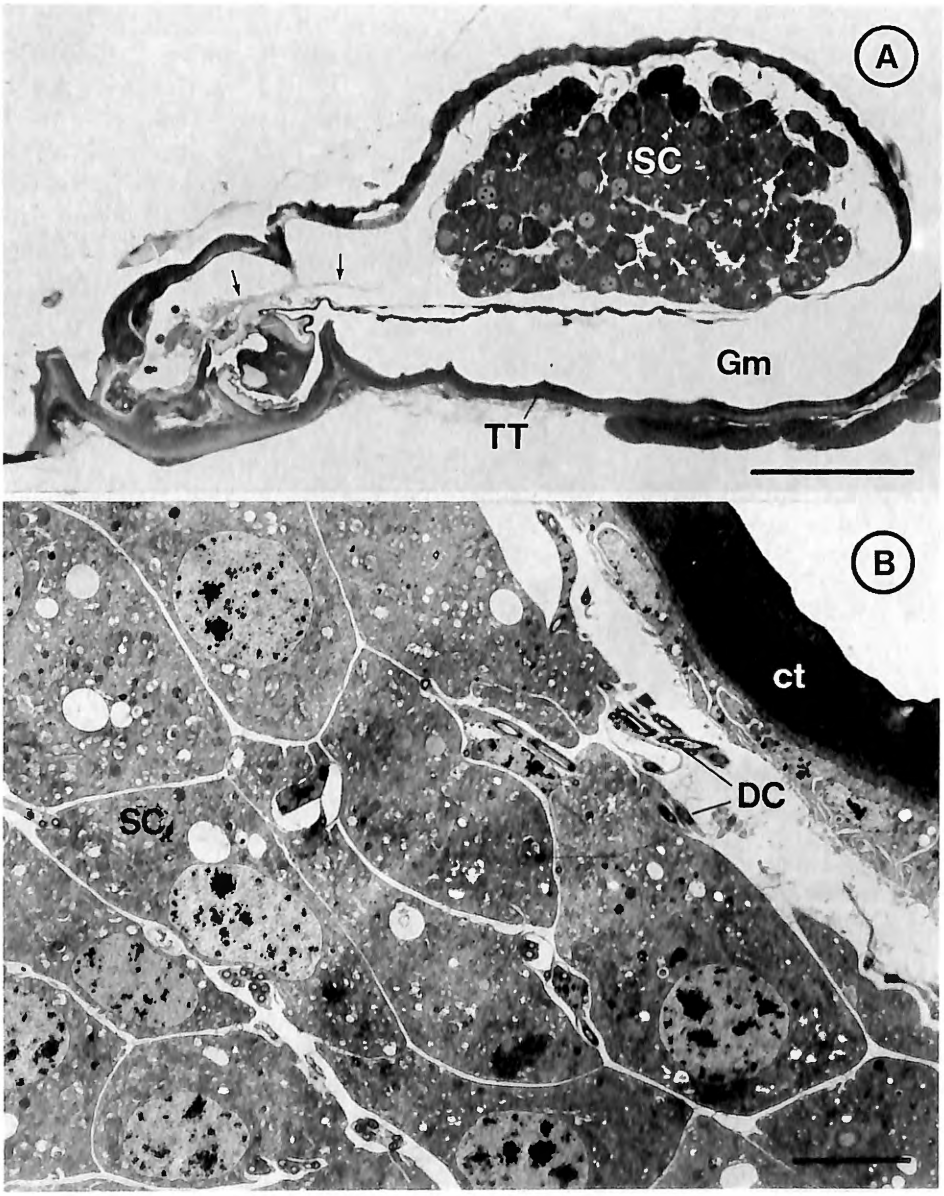
PLATE I

Scanning electron micrograph of left side gemma, showing its natural position (anterior side to the left; scale bar 100 μm).

Each secretory cell is associated with a slender duct cell, that forms the connection between the end apparatus and the external environment. Within the duct cell, one narrow duct with a diameter around 0.4 μm is found with a continuous cuticular lining having a uniform thickness of 0.1 μm. The cytoplasm is very much reduced and forms a narrow sheath around the duct (Pl. III, D). All ducts open through pores with a diameter around 0.3 μm in the wrinkled outer surface of the gemma (Pl. III, E). The length of the duct cells varies from as less as 20 μm for those associated with secretory cells near the dorsal surface, to about 150 μm for those originating from cells near the ventral wall.

Apart from the abundance of gland cells and their ducts, the gemmae contain only very few tracheoles and a nerve fibre (Pl. III, F), that penetrate from the thorax through the stalk. Not a single muscle fibre could be detected inside the gemmae.

PLATE II



DISCUSSION

The existence of a large number of glandular cells inside the gemmae of *Diacamma australe* make these thoracic appendages an important exocrine organ. Although the gemmae are homologous to mesothoracic wings (TULLOCH, 1934 ; BITSCH and PEETERS, 1991), they are highly modified, and the de novo evolution of this exocrine structure is characteristic of the genus *Diacamma*. The cellular organization of the gland is consistent with that of bicellular secretory units, each comprising a secretory cell and a duct cell (BILLEN, 1987). The contact area between these cells is the highly-specialized end apparatus, which regulates the discharge of secretory products at the cellular level (NOIROT and QUENNEDEY, 1974 ; BILLEN, 1990). The microvillar arrangement in the end apparatus may vary considerably, even between the secretory cells of the same individual, because of the frequent appearance of conspicuous spaces in this area. These spaces, in spite of their apparent position within the secretory cell, do represent the extracellular area, since the cell membrane (of the microvilli) lining them is nothing but the invaginated plasmalemma of the secretory cell. The occurrence of these spaces, which are commonly found in exocrine glands of the type having an end apparatus (BILLEN, 1990), probably corresponds to the creation of additional volume for the storage of secretions (BAZIRE-BENAZET and ZYLBERBERG, 1979).

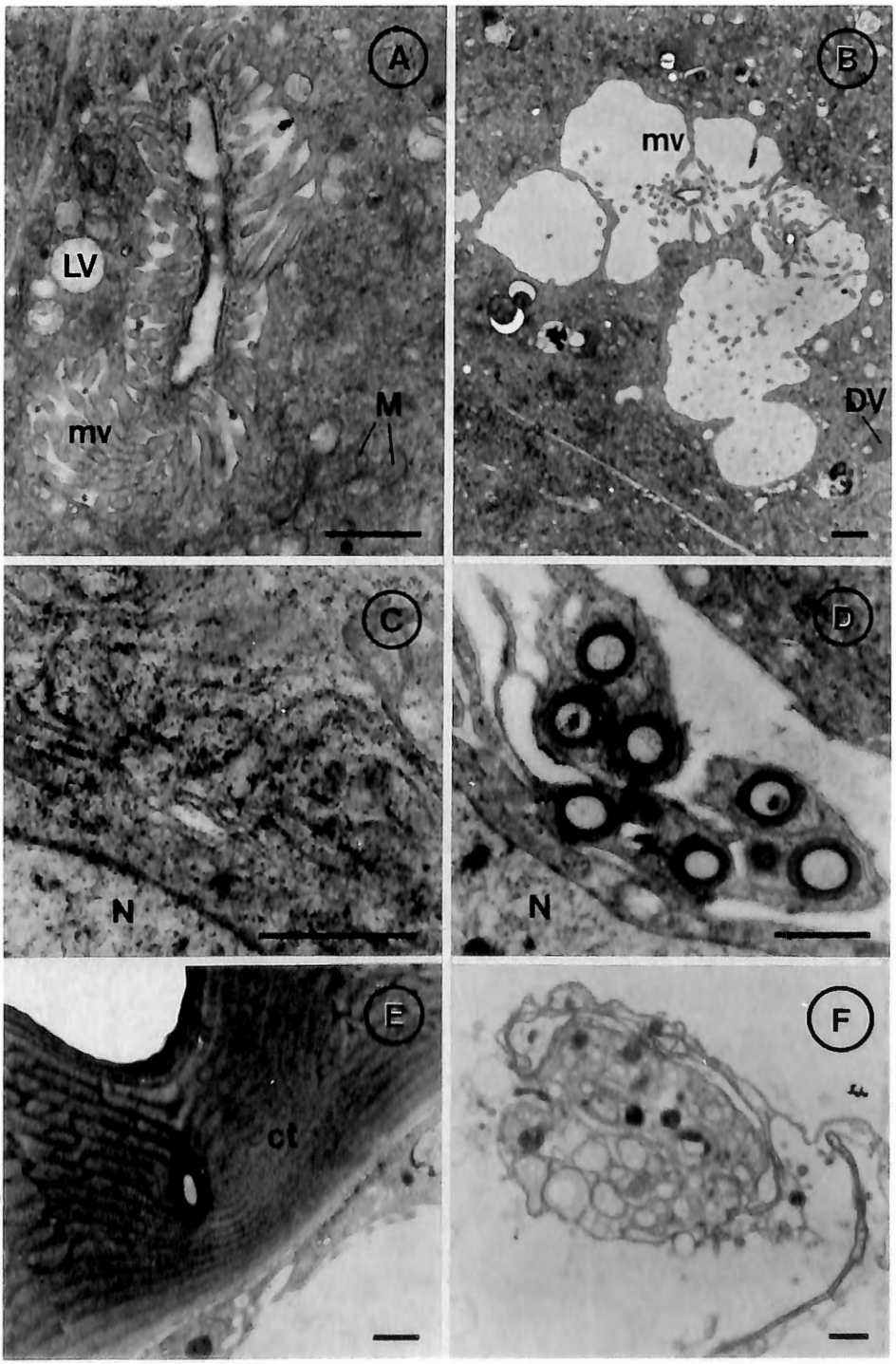
No information is yet available on the chemical nature of the gemmae secretions. The occurrence of both smooth and granular endoplasmic reticulum, as well as both electron-lucid and electron-dense (secretory ?) vesicles, suggests that a complex mixture may be produced. Behavioural data indicate that the secretions may be volatile. The presence of a nerve fibre into each gemma possibly is to be related with the existence of a stretch receptor in the gemmae. This would be a plausible mechanism for detecting when a gemma has been mutilated, with eventual subsequent neuroendocrine effects leading to behavioural changes. The lack of any kind of musculature inside the gemmae, which in itself is consistent with the proposed homology with wings, seems to rule out the possibility that the secretions are released under voluntary control. Therefore, a process of passive diffusion may result in the continuous release of secretions.

PLATE II

A. Semithin longitudinal section through the gemma in a callow worker of *D. australe* (anterior side to the left ; scale bar 10 µm). Note the penetration of a nerve fibre (arrows) through the stalk of the gemma and the difference in thickness of the cuticular lining between the gemma's outer and inner wall. Gm = gemmarium, SC = secretory cells, TT = thoracic tegument.

B. General ultrastructural view of the secretory cells (SC) and duct cells (DC). ct = cuticular lining of outer side. Scale bar 10 µm.

PLATE III



The numerous secretory units release their products to the external environment via pores on the outer surface of the gemmae. Behavioural observations suggest that these pheromones are involved in the social interactions among workers (FUKUMOTO *et al.*, 1989 ; PEETERS and HIGASHI, 1989). Further work is necessary to determine whether they also function as sexual pheromones to attract males.

ACKNOWLEDGEMENTS

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PLATE III

Ultrastructural details of the gemma in a callow worker, scale bar 1 µm in all figures.
A. end apparatus with closely arranged microvilli ; cytoplasm with mainly smooth endoplasmic reticulum. B. end apparatus with distorted microvillar arrangement. C. cytoplasm with mainly granular endoplasmic reticulum. D. section through bundle of duct cells. E. duct penetrating through thick upper tegument of gemma. F. section through nerve fibre in stalk area.

ct = cuticle, DV = electron-dense vesicle, LV = electron-lucid vesicle, M = mitochondria, mv = microvilli, N = nucleus.

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POTENTIAL TAXONOMIC APPLICATIONS OF H.P.L.C. ANALYSIS OF COCCOIDEA PIGMENTS (HOMOPTERA : STERNORHYNCHA)

by

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SUMMARY

High Performance Liquid Chromatography (HPLC) was applied to separate and identify the constituents of the pigments of scale insects. We concentrated on the following problems : the classification of unsufficiently characterised Near-Eastern Kermesidae (*Kermes biblicus* and *K. palestiniensis*) ; the relationship between *K vermilio* and *K. ballotae*, living on the same plant species ; and the search for complementary evidence for a suggested identity of *Porphyrophora polonica* and *P. crithmi* (Margarodidae).

It was shown that the pigment composition of Near-Eastern Kermesidae might be helpful in further taxonomic studies. The differences between the pigments of *K. vermilio* and *K. ballotae* contradict their being only forms of the same species. The pigment composition of both *Porphyrophora* species studied is exactly the same.

Keywords : HPLC, pigments, chemotaxonomy, *Kermes, Porphyrophora.*

INTRODUCTION

Recently, the High Performance Liquid Chromatography (HPLC) analysis of pigments present in some species of scale insects of the superfamily Coccoidea has been worked out (WOUTERS and VERHECKEN, 1989). The main purpose of that study was to investigate the dye contents of the few species of *Kermes, Dactylopius, Porphyrophora* and *Kerria* which have been used historically for textile dyeing purposes. From this study it followed that dye analysis allows the species-level identification of the taxa studied, even when analysing only their alcoholic preserving fluids (WOUTERS, 1990). Most of this work was done on adult female insects. It was found that the pigments are present in the insect as free anthraquinone dyestuffs and/or as dyestuff precursors, probably anthraquinone glycosides.

During cited work, we came across a number of species for which the exact taxonomic status is not clearly defined. The present paper reports on the pigment content of these species. It is hoped that this new information may be useful to entomologists as an extra tool, possibly enabling them to gain a better understanding of the identity of these species and of the taxonomic relations between them.

MATERIALS AND METHODS

Samples

Specimens of a *Kermes* species collected at Kibbutz Eilon and nearby Adamit on the Lebanese border of Israel in June 1987, and identified as *Kermes biblicus* BODENHEIMER, 1926, by prof. Sternlicht, were obtained from dr. I. Ziderman (Israel Fiber Institute, Jerusalem). First instar larvae from this sample were identified by D. Matile-Ferrero (MNHN, Paris) as probably belonging to *K. palestiniensis* BALACHOWSKY, 1953, with the remark : « en l'absence d'éléments concrets nous permettant d'identifier correctement toutes les espèces de *Kermes* du bassin Méditerranéen, je ne tiens compte actuellement que des espèces accompagnées d'une description moderne et détaillée et notamment du travail de BALACHOWSKY (1953) ». This sample is here referred to as *K. biblicus,* not implying however the acceptance of this name as the valid one for the taxon.

Samples collected by the second author at Harbiye (southern Turkey, near Antakya) and Fethiye (western Turkey) in June 1989, were visually clearly different from each other because of the parallel black bands on the material from Fethiye. The latter might belong to the species provisionally named *Kermes virgatus* CARDON, 1990, cited for western and southwestern Turkey. Both our Harbiye and Fethiye samples were identified on the first instar larvae by D. Matile-Ferrero as *K. palestiniensis,* with the same restrictive remark as for the Israeli sample.

Kermes vermilio (PLANCHON, 1864) and *K. ilicis* (LINNÉ, 1758) were collected in Southern France by the second author (VERHECKEN and WOUTERS, 1990).

Specimens of *Kermes ballotae* SIGNORET, 1874, from the Pisa region (Italy) were donated by D. Matile-Ferrero.

Cysts and adult specimens of *Porphyrophora polonica* (LINNÉ, 1758), both before and after ovipositing, were given by E. Szymula (Jagellonean University of Krakow, Poland). The were found on the roots of *Scleranthus perennis* in the vicinity of Czestochova in May 1990.

Adult females of *Porphyrophora crithmi* (GOUX, 1938) from the South of France were donated by Dr. I. Foldi (MNHN, Paris) ; they were described as being fecondated.

Preparation of samples

It is preferred to analyse insects that were not killed by immersion in solvents and that were not put in a preserving liquid, to avoid any loss of pigments before

analysis. Some species (*e.g. Porphyrophora*) are treated in a delipidating solvent (methanol/chloroform, 2/1, v/v) which was shown to extract no pigment, to improve their subsequent aqueous extraction.

One to three air-dried insects are finely powdered and extracted in water/methanol (9/1, v/v) for various periods of time at room temperature or at the boil. The extracts are cleared under centrifugal force. Mild acid hydrolysis is performed to convert any acid-labile precursor to the actual pigment. Extracts are dried in a dessicator and the residues are dissolved in an appropriate volume of water/methanol (1/1, v/v) just before analysis. When the pigment content of these preparations is too low, they are absorbed on alum mordanted wool and recovered by acid hydrolysis (see Results).

Analytical procedure

High Performance Liquid Chromatography (HPLC) allows both separation and quantification of all the dye components present. Computerized diode-array detection makes it possible to analyse a sample in one single run at any wavelenght between 200 and 800 nm, i. e. the complete ultraviolet and visible spectrum. All the analytical data are stored on a computer hard-disk, from which they can be retrieved, printed and backed-up. A schematic representation of the analytical equipment is given by WOUTERS (1990). For the separation a column is used, 4.6 × 100 mm, filled with a reversed-phase adsorbent (Spherisorb ODS2, Biorad-RSL, Belgium). Typically 20 µl are injected and the column is then eluted with a concentration gradient of methanol in water, in the presence of phosphoric acid. Elution of one sample takes 30 minutes. At an eluent flow of 1.2 ml/min a system back pressure of 200 bar is generated. Further details are given in WOUTERS and VERHECKEN (1989).

RESULTS AND DISCUSSION

Selection of the way of presentation

The pigment content of scale insects known to have been used as dye sources in the past, usually varies between 1 and 20 % of the female body mass. Aqueous extracts of these species contain enough dye to be chromatographed as such (WOUTERS and VERHECKEN, 1989). At very low concentration however, individual dye components may be undetected in aqueous extracts and therefore other techniques must be applied.

Figure 1a gives the chromatogram, recorded at a wavelength of 275 nm, of an aqueous extract of *Kermes ilicis*. This wavelength ensures the highest possible sensitivity for most insect dyes (WOUTERS and VERHECKEN, 1989). Figures 1b and 1c depict the same analysis, presented however at 420 nm and 500 nm, in order to detect yellow and red dyes, respectively. It is clear that the dye content of the aqueous extract must be very low and that individual dye components may not be characterised properly.

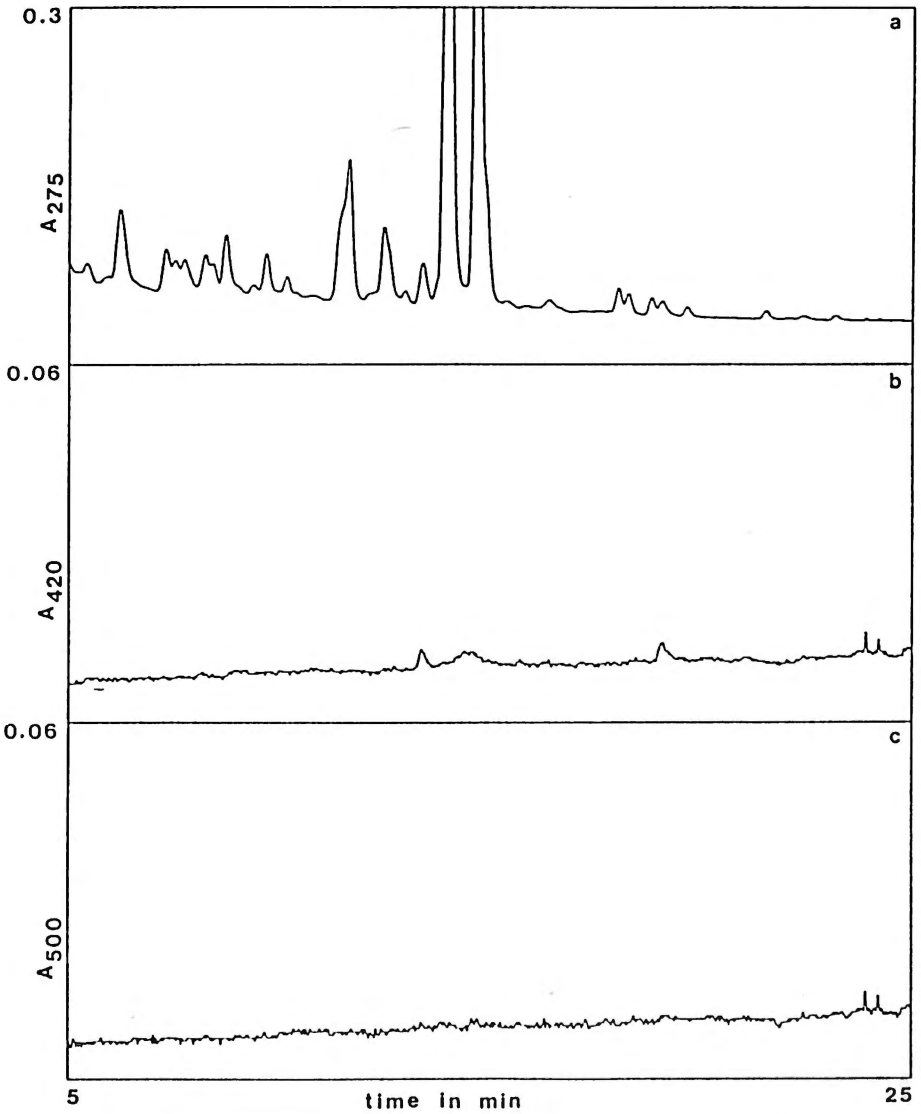

Fig. 1. — HPLC of *Kermes ilicis* pigments, monitored at (a) 275 nm, (b) 420 nm and (c) 500 nm. Aqueous extract.

In Figures 2a-c are given the chromatograms, taken at 275 nm, 420 nm and 500 nm, of an acidic hydrolysate of the aqueous extract, using exactly the same scaling as in Fig. 1a-c. It is obvious that the recovery of dyestuff is higher, probably due to hydrolysis of precursors.

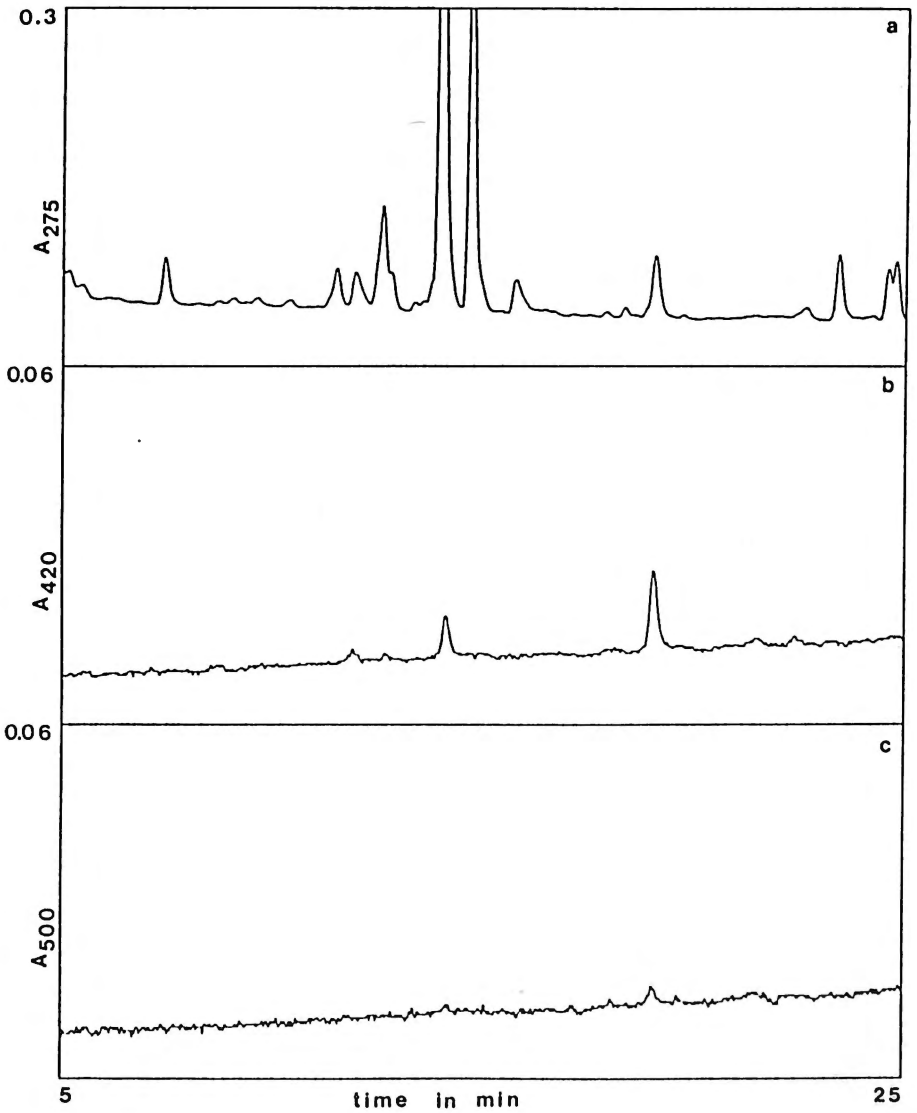

Fig. 2. — HPLC of *Kermes ilicis* pigments, monitored at (a) 275 nm, (b) 420 nm and (c) 500 nm. Acid hydrolysate of aqueous extract.

Figs 3a-c give the chromatograms, using exactly the same parameters as in Figs 1a-c and 2a-c, of a dye sample obtained from a wool yarn dyed with *K. ilicis*. The individual dye components are clearly recognisable now and their detection sensitivity is sufficient for spectral characterisation and quantification. The wool-dyeing experiment, originating from our previous work (WOUTERS and VERHECKEN,

1989a), increases the dye content of the samples to be analysed because the dyes are selectively taken up by the wool.

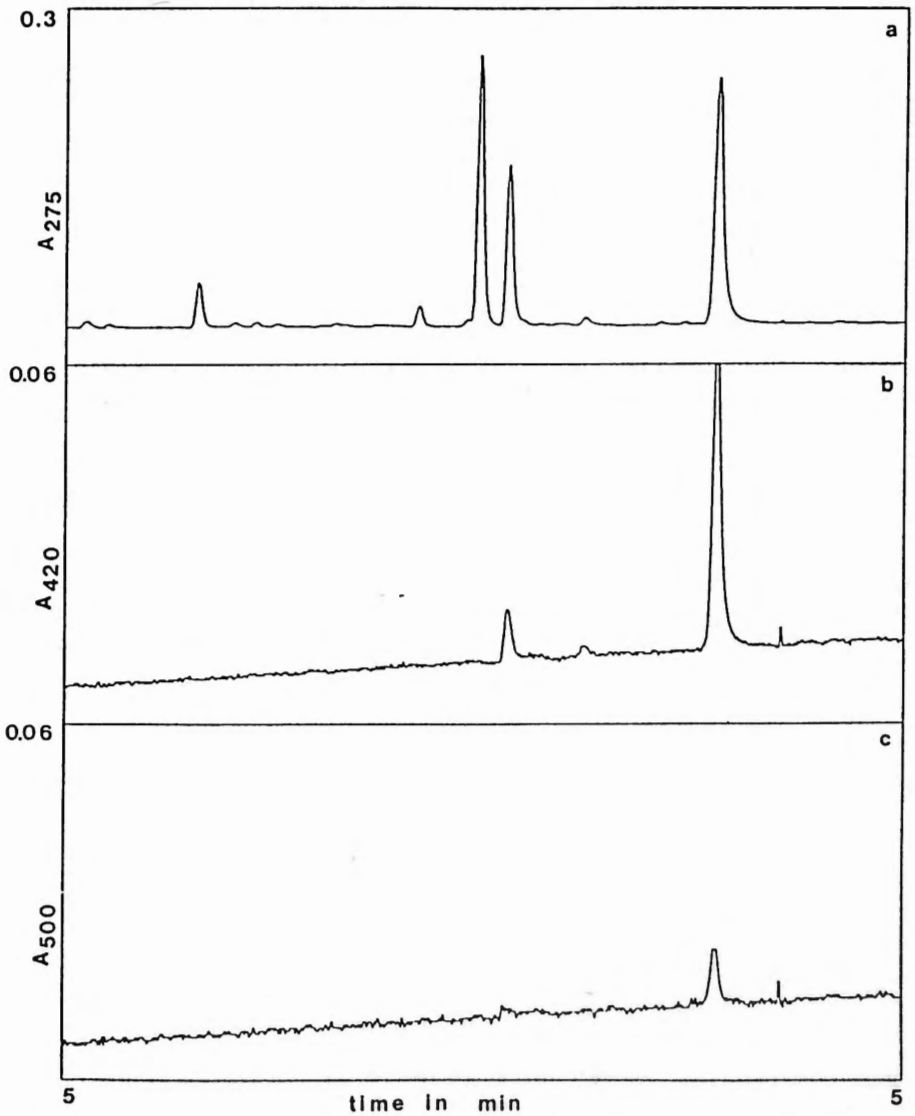

Fig. 3. — HPLC of *Kermes ilicis* pigments, monitored at (a) 275 nm, (b) 420 nm and (c) 500 nm. Pigments recovered from dyed wool.

Kermes biblicus, K. palestiniensis* and *K. ilicis

Kermes biblicus was described for adult specimens from Syria (actual Lebanon, see BEN-DOV and HARPAZ, 1985) ; BODENHEIMER (1931) later also introduced one species from Anatolia and two from Palestine. While reviewing the *Kermes* species of the eastern Mediterranean basin, BALACHOWSKY (1953) was unable to identify these species, since their description was only based on old adult females and not on first instar larvae, and Bodenheimer's type material could not be located. BALACHOWSKY (1953) thought it had disappeared (it is not in the Bodenheimer Collection, Hebrew University of Israel, Rehovot (BEN-DOV and HARPAZ, 1985), but it was reported recently to be in the American University in Beyruth (CARDON, 1990)) and therefore introduced three new species from Israel : *Kermes echinatus, K. spatulatus* and *K. palestiniensis*. He also stated that some of them might be identical with one of BODENHEIMER's species, and (BALACHOWSKY, 1950) that the description of *K. biblicus* might as well apply to *K. ilicis* LINNÉ.

STERNLICHT (1969) described another species for Israel : *Kermes bytinskii*. Study of specimens (not types ?) from Bodenheimer's collection (on loan from BMNH, London, and from prof. Harpaz (whereabouts not stated) led him to surmise, among others, that *K. palestiniensis* and *K. biblicus* are identical.

It is not clear why STERNLICHT (see samples) preferred the name *K. biblicus* to *K. palestiniensis* for the identification of the Israeli sample studied here since, while describing *K. bytinskii,* he implicitly considered BODENHEIMER's (1926, 1931) names to be *nomina nuda,* and in a later paper (STERNLICHT, 1980) he did not use the latter author's names for the two other Israeli species. Moreover, in 1980 he described the crawlers of *K. biblicus* as being red ; this was not the case in the specimens we received under that name. Preliminary tests did not show evidence of a red dye in this sample (VERHECKEN and WOUTERS, 1990). This is in contradiction with results obtained by TAYLOR (1988) by means of paper chromatography of a sample from the same source (but of the same identity ?) ; he suggests the presence of anthragallol and ceroalbolinic acid.

The pigments of the three species were concentrated on wool, since they were present in very low amounts. It is emphasised that the recovery of the dyes from the wool is always done in exactly the same way (WOUTERS and VERHECKEN, 1989a). The chromatograms obtained for *K. ilicis, K. biblicus, K. palestiniensis* (unstriped specimen from Harbiye) and *K. palestiniensis* (striped specimen from Fethiye) are given in Fig. 4a-d, respectively. The detection wavelength was 420 nm since no red pigments were shown to be present in a preliminary search. The identification of ceroalbolinic acid (caa) (GADGIL *et al.,* 1968) was made according to the presence of this peak in an acid extract of *Ceroplastes rubens* MASKELL, 1893 (WOUTERS, unpublished results), and to the data in BANKS (1977). Flavokermesic acid (fk) and kermesic acid (ka) were characterised previously (WOUTERS and VERHECKEN, 1989). The chemical structure of the three other peaks is unknown, therefore these are designated K1, K2 and K3 ; « K » from Kermesidae and the figure following the elution order. According to the chemical pathway presented by BROWN (1975), there should be a link between fk, desoxyerythrolaccin and both erythro- and

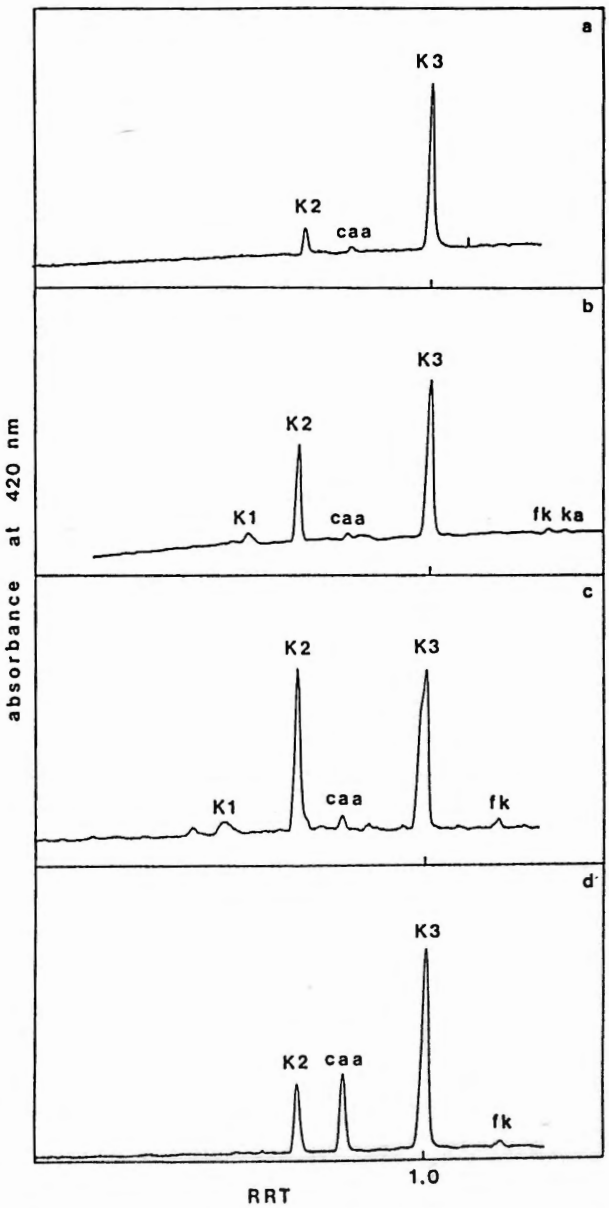

Fig. 4. — HPLC of Kermesidae pigments, recovered from dyed wool, monitored at 420 nm. (a) : *Kermes ilicis*; (b) : *Kermes biblicus*; (c) : *K. palestiniensis*, unstriped specimen; (d) : *K. palestiniensis*, striped specimen. The abbreviations refer to table 2. RRT : retention time, relative to K3 (RRT of K3 = 1.00). Small variations in RRT of fk and ka in (b) are due to modification of the elution program.

isoerythrolaccin. The identity of either of the latter products with K1, K2 or K3 could not be established because of the lack of the reference products, and since it was not clear whether or not the minor differences between the spectra we recorded and those given by THOMSON (1971) were due to the different solvents used. The two most related samples are *K. biblicus* and *K. palestiniensis* (unstriped).

The present findings support STERNLICHT's (1969) hypothesis regarding the possible identity of *K. palestiniensis* (in this case the unstriped specimen) and *K. biblicus*. Both *K. ilicis* and *K. palestiniensis* (striped) seem to be more divergent from the former and from each other : in *K. ilicis* only K3 is abundantly present ; in the striped *K. palestiniensis* important amounts of K2 and caa were also found, as well as some fk.

The occurrence of K1, K2 caa, K3 and fk in varying combinations suggests a close relationship of the species studied, and a particular position of this species group within the Kermesidae. It is very unlikely that scale insects with either of the four dye compositions found here may produce a red colour on wool. The only red component, ka, was found in *K. biblicus* but in so tiny an amount that its contribution to colour is negligible.

The two main components for *K. biblicus,* K2 and K3, may refer to the two spots obtained by paper chromatography of an acid insect extract (TAYLOR, 1988). However, the suggested identity of one of them with anthragallol may be ruled out definitely.

Kermes vermilio and *K. ballotae*

K. vermilio was described for the species living on the kermes oak *Quercus coccifera* and yielding the red dye historically used for dyeing textiles. *K. ballotae* is considered by BALACHOWSKY (1950a) as a form of *K. vermilio* living on *Quercus ilex* and *Q. suber*. He gives distinguishing features of adult females ; he states that their neonate larvae are strictly identical and consequently he understands both « forms » as belonging to a single polymorphous species. He never found *K. ballotae* on *Q. coccifera,* nor *K. vermilio* on *Q. ilex,* and concluded that the polymorphism and heterochromy are due to the habitat.

According to information obtained from D. Matile-Ferrero, both *K. vermilio* and *K. ballotae* are now reported to live on *Quercus coccifera* and on *Q. ilex* ; this changes the concept of their being only forms of the same species.

The detailed dye analysis of the females of *K. vermilio* was reported earlier (WOUTERS and VERHECKEN, 1989). In an extract of *K. vermilio,* three dyestuffs were obvious : kvI, fk and ka. Acid hydrolysis destroys kvI and generates both fk and ka. It was possible to reveal two components in kvI : kvIa and kvIb. Their spectrum suggests them to be precursors for fk and ka, respectively (WOUTERS and VERHECKEN, 1989). The relative abundances of fk and ka were 33/67 in the extract, 26/74 in the acid hydrolysate, and 14/86 in the sample prepared from dyed wool.

In the extract of *K. ballotae,* four dyestuff components were detected : kbaI, kbaII, fk and ka. Both kbaI and kbaII are hydrolysed in acid to fk. The relative

abundances of fk and ka in the extract were 63/37; after acid hydrolysis this changes into 75/25, and to 82/18 for a sample from dyed wool. Distinction with *K. vermilio* may be made either by analysis of the precursors or by considering the fk/ka ratio, the latter preferentially after acid hydrolysis.

The present data allowed us to reconsider the identity of a sample of crushed *« Kermes vermilio »* sent to us by Prof. Boyer (CNRS, Draguignan, France) several years ago. A HPLC analysis of it was already published (WOUTERS, 1985). It is now known that the large peak preceding ka in that chromatogram is fk, so that the identify of the sample must be *K. ballotae* rather than *K. vermilio*. Probably, this material had been identified according to its habitat, rather than on entomological features.

Porphyrophora polonica and *P. crithmi*

Porphyrophora polonica lives on roots of some plant species in Central and Eastern Europe. *P. crithmi* was described as a geographical subspecies of the North-African *Margarodes buxtoni* NEWSTEAD, 1917, for specimens living at Marseille on subterranean parts of *Crithmum maritimum*. It is judged to be identical, even at cytological level, with *P. polonica* by Dr. I. Foldi (CARDON, 1990), who requested us to check his findings by pigment analysis.

A chromatographic solution to this problem was not straighforward since it was known that the pigment composition of *P. polonica* varies with its development stage (WOUTERS and VERHECKEN, 1989) : at least seven components, including two acid-labile precursors, were present in varying ratios. The *P. crithmi* females sent by Dr. Foldi were described as follows in the accompanying letter : « 1 tube avec six

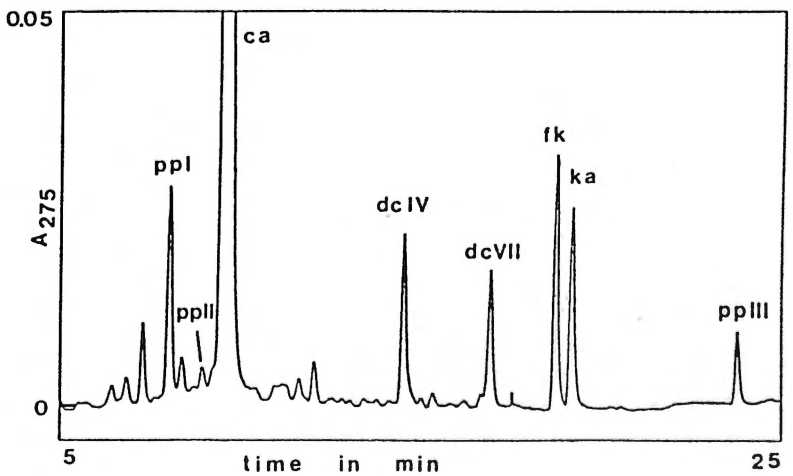

Fig. 5. — HPLC of an aqueous extract of *Porphyrophora crithmi* pigments (adult females, fecondated), monitored at 275 nm. Abbreviations refer to table 2.

TABLE 1

Dyestuff composition of *Porphyrophora polonica,* females (pp/f), in following stages : cyst (/c), before ovipositing (/bo), ovipositing (/o) ; and of *Porphyrophora crithmi,* females (pc/f), fecundated (/f). The addition /h points to a sample hydrolysed in acid. Figures represent integration values, taken at 275 mm ; figures between brackets represent relative ratios within the group of components considered.

Sample	ppI	ppII	ca	dcIV	dcVII	fk	ka	ppIII
pp/f/c	7.6	32	139 (86.9)	1.4	1.4	2.1 (1.3)	19 (11.9)	—
pp/f/c/h	—	—	123 (69.1)	3.2	2.7	9.1 (5.1)	46 (25.8)	—
pp/f/bo	13	29	258 (96.6)	4.6	2.2	2.3 (0.9)	6.7 (25)	—
pp/f/bo/h	—	—	226 (82.2)	5.7	5.0	13 (4.7)	36 (13.1)	0.4 (0.1)
pp/f/o	6.6	0.6	139 (97.9)	1.9	1.4	2.2 (1.5)	0.2 (0.2)	—
pp/f/o/h	—	—	114 (92.7)	3.0	2.5	7.6 (6.2)	1.3 (1.0)	0.3 (0.2)
pc/f/f	3.8	0.3	197 (95.2)	3.6	2.5	4.6 (2.2)	3.9 (1.9)	1.2 (0.6)
pc/f/f/h	—	—	177 (92.2)	4.6	4.2	8.4 (4.4)	4.5 (2.3)	2.3 (1.2)

femelles : la secrétion blanchâtre signifie qu'elles ont été fécondées ». Only recently we obtained several well identified lots of *P. polonica,* described as follows : « 1. female cysts ; 2. adult females before ovipositing ; 3. adult females, ovipositing ». The analytical results obtained from aqueous extracts and from acid hydrolysed extracts are given in Table 1. For clarity, one chromatogram is depicted also (Fig. 5), wherein each considered is designated by its abbreviation.

The *P. polonica* cysts were clearly distinct from the other samples in showing higher amounts in ka, especially after acid hydrolysis, and in being devoid of ppIII. A striking similarity occurs between *P. polonica* (females, ovipositing) and *P. crithmi* (females, fecundated) : in both cases fk prevails over ka, and the amounts of these latter compounds are relatively low compared to the other samples ; the presence of ppIII in the hydrolysed samples is also common to both samples ; and the ratio ppI/ppIII is the same in the preparations considered. Acid hydrolysis destroys ppI and ppII in all samples and an almost quantitative transition between either ppI and fk or ppII and ka can be seen, thus proving that ppI is the precursor of fk, and ppII the one of ka.

According to the pigment analyses, *P. polonica* is identical to *P. crithmi.* The entomological implication of the chemical data is not in contradiction with the distribution area of *P. polonica,* as described by KOSZTARAB and KOZÁR (1988) and by YASCHENKO (1990).

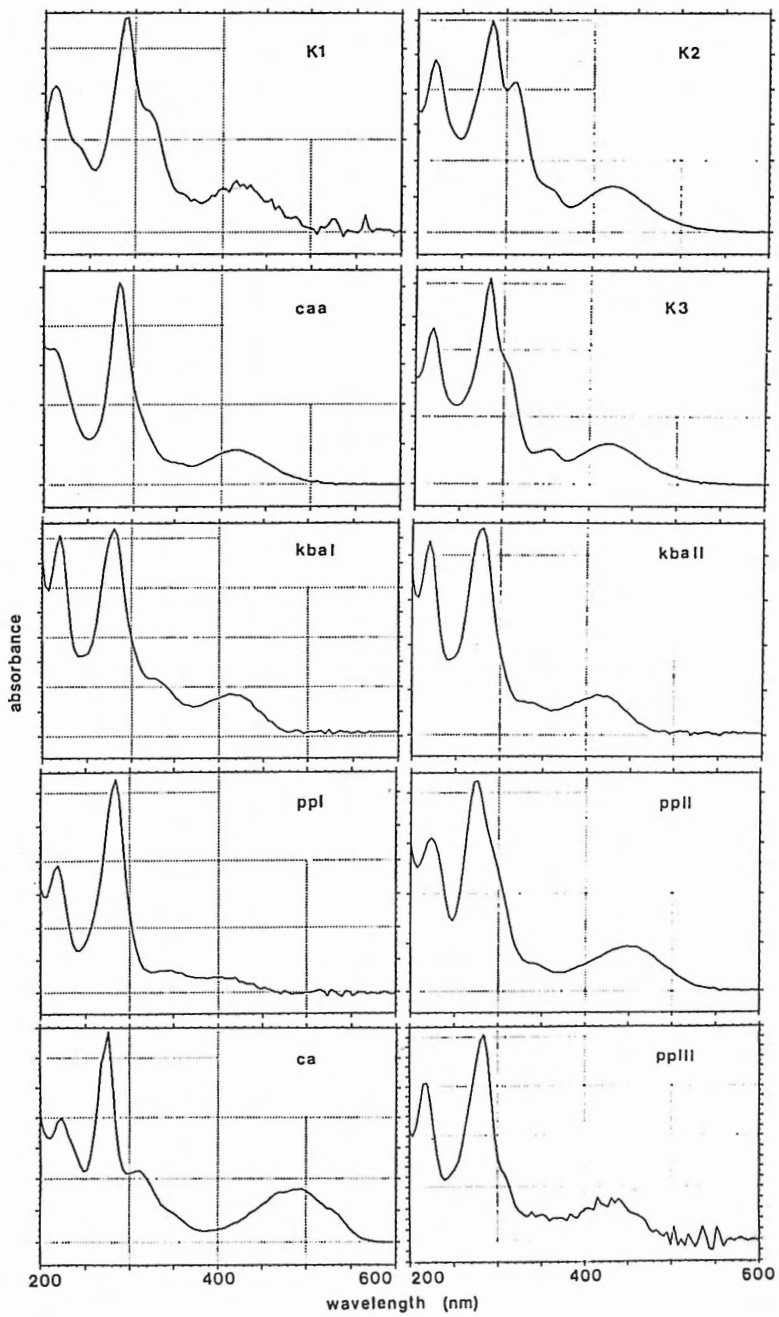

Fig. 6. — Spectral characterisation of individual pigment components from scale insects studied. Abbreviations refer to Table 2.

TABLE 2

Nomenclature, abbreviations, spectral characterisation and relative retention time in described HPLC conditions of the scale insect pigment components. (*) refers to the present paper ; W & V to WOUTERS and VERHECKEN, 1989.

unknown yellow	K1	(*)	1.02
unknown yellow	K2	(*)	1.18
ceroalbolinic acid	caa	THOMSON, 1971	1.33
unknown yellow	K3	(*)	1.59
flavokermesic acid	fk	W & V Fig. 11 e	1.98
kermesic acid	ka	W & V Fig. 11 f	2.03
fk precursor in pp	ppI	W & V Fig. 11 i	0.85
ka precursor in pp	ppII	(*)	0.95
carminic acid	ca	WOUTERS, 1985	1.00
unknown yellow	ppIII	(*)	2.51
fk precursor in kv	kvIa	W & V Fig. 11 g	1.34
ka precursor in kv	kvIb	W & V Fig. 11 h	1.38
unknown red	dcIV	W & V Fig. 11 c	1.52
unknown red	dcVII	W & V Fig. 11 d	1.78
fk precursor in kba	kbaI	(*)	1.32
fk precursor in kba	kbaII	(*)	1.34

CONCLUSION

The high-level analytical capabilities of HPLC allowed us to characterise and quantitate several pigment constituents in scale insects. The combination of the (relative) retention time of a component (Table 2) with its absorption characteristics in the ultraviolet and visible region of electromagnetic radiations (Fig. 6) was used to trace common compositional features in related species, even when the exact chemical nature of a given compound was not known. It should be born in mind however that chemotaxonomic considerations should only be given in close collaboration with entomologists. For example, the striking similarity between *Porphyrophora polonica* and *P. crithmi* is only visible when comparing specimens in the same biological condition. This statement implies that the chromatographer should be aware of possible variations in pigment composition of scale insects as a function of their developmental stage. The detection of these variations, implying the absolute necessity of separation, characterisation and quantification of the pigment components, can at present only be performed by means of HPLC.

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CINERADIOGRAPHIC ANALYSIS OF THE PHARYNGEAL JAW MOVEMENTS DURING FEEDING IN *HAPLOCHROMIS BURTONI* (GÜNTHER, 1893) (PISCES, CICHLIDAE)

by

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SUMMARY

Analysis of lateral radiographic films reveals that the upper and lower pharyngeal jaws in *Haplochromis burtoni* (GÜNTHER, 1893) show opposite anteroposterior displacements during food reduction. Force is generated during upper jaw retraction by elevation and slight protraction of the lower pharyngeal jaw (compression phase) and subsequently by depression and strong protraction of the lower jaw (shearing phase). These results contradict previous findings in which the upper and lower pharyngeal jaws show synchronous protraction and retraction.

Keywords : Cichlids, feeding, pharyngeal jaws.

INTRODUCTION

The pharyngeal jaw apparatus of cichlids is (amongst that of some other teleostean families) characterized by two upper pharyngeal jaws, which articulate with the neurocranial base, and a single lower pharyngeal jaw (LIEM and GREENWOOD, 1981). Although it is suggested that a powerful upper-lower pharyngeal jaw bite exists (LIEM and GREENWOOD, 1981), experimental studies of the mechanisms of pharyngeal mastication are very scarce.

The first experimental study on pharyngeal mastication in cichlids (LIEM, 1973) emphasized muscle activities in *Haplochromis burtoni* (GÜNTHER, 1893) (an insectivorous-omnivorous cichlid, see JANSSENS DE BISTHOVEN *et al.*, 1990) during feeding on *Gammarus* species ; positions of the pharyngeal jaws during mastication were inferred from successive X-ray pictures. LIEM (1973) described a triphasic movement cycle in which the upper and lower pharyngeal jaws are protracted and retracted simultaneously. This pattern was confirmed by electromyographical and cineradiographical study on piscivorous cichlid species (LIEM, 1978), and subsequently generalized for all cichlid species (LIEM and GREENWOOD, 1981).

Recent cineradiographic studies (AERTS *et al.*, 1986 ; CLAES and DE VREE, 1989, 1991) revealed that pharyngeal food processing in *Oreochromis niloticus* (LINN, 1758) involves opposite anteroposterior movements of the upper and lower pharyngeal jaws. Preliminary results on *Cichlasoma friedrichsthali* (HECKEL, 1840) (a Central-American carnivorous cichlid) and *Astatoreochromis alluaudi* (PELLEGRIN, 1903) (a haplochromine durophagous species) confirm this « opposite movement » pattern. Since *Oreochromis, Astatoreochromis* and *Cichlasoma* represent different lineages of the family (GREENWOOD, 1978), these results suggest that the alternating pattern is probably the most common in cichlids.

However, a comparison between these quantitative results and the movement profiles described by LIEM (1973, 1978) is difficult, since LIEM offers only a qualitative impression of the pharyngeal jaw movements in *Haplochromis burtoni* (LIEM, 1973) and piscivorous cichlids (LIEM, 1978). Therefore, a detailed quantitative re-examination of the kinematic pattern in these species is needed. In the present study, we report the results of a cineradiographical analysis of the pharyngeal jaw movements in *Haplochromis burtoni.*

MATERIAL AND METHODS

Two individuals of *Haplochromis burtoni* (GÜNTHER, 1893), purchased from a commercial supplier, were trained to feed on live crickets and earthworms in a

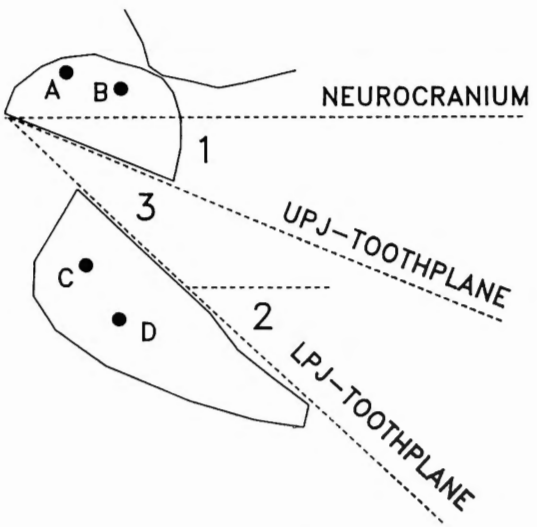

Fig. 1. — Position of the upper (A,B) and lower (C,D) pharyngeal jaw markers. Calculated angles are also indicated : between the upper pharyngeal jaws and the neurocranium (1), the lower pharyngeal jaw and the neurocranium (2) and between the upper and lower pharyngeal jaws (3).

narrow (6 cm) experimental aquarium tank, provided with thin (2 mm) plexiglass on the lateral walls. Lateral X-ray films (Gevapan 30 negative film, 80 ASA) were taken at 50 frames per second with an Arriflex 16 mm camera, attached to a Sirecon 2 image amplifier; X-rays were generated by a Siemens Tridoros 800 Optimatic at 1 meter distance from the image amplifier. All food items were impregnated with a solution of barium sulphate in order to make their position visible on the X-ray films. Small lead markers were implanted under anesthesia (MS 222) in the pharyngeal jaws and the neurocranium. The marker coordinates

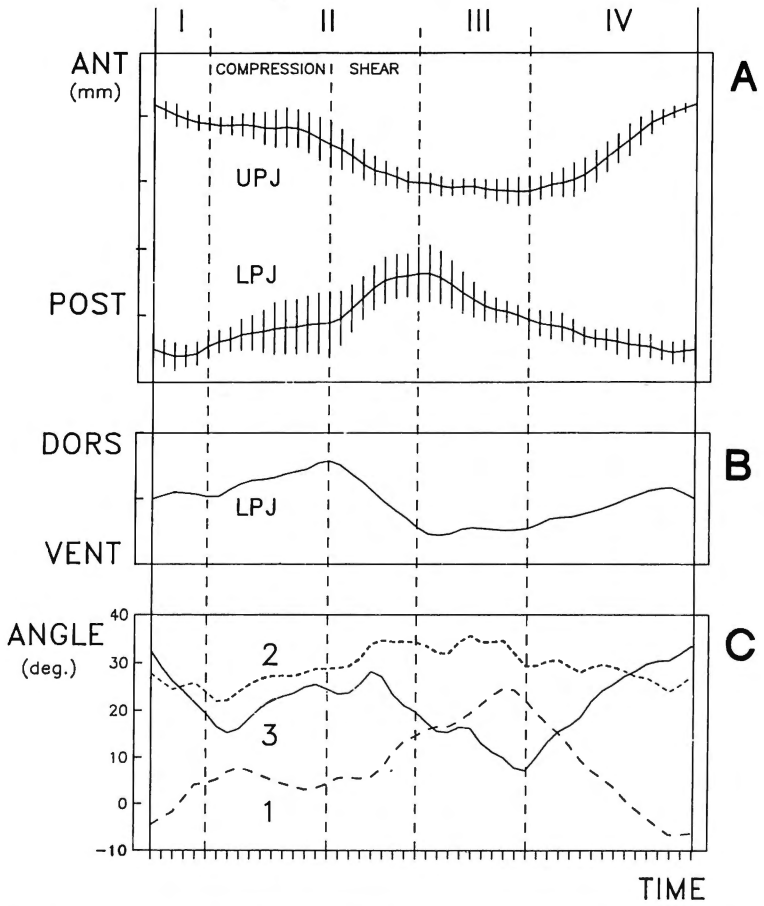

Fig. 2. — Time-displacement graph for a mean movement cycle during pharyngeal processing of an earthworm (N = 11). Upper and lower jaw movements are indicated by the displacements of marker A and C resp. (see Fig. 1). ANT, anterior; POST, posterior; DORS, dorsal; VENT, ventral; UPJ, upper pharyngeal jaw; LPJ, lower pharyngeal jaw. Angles are indicated in Fig. 1. I = preparatory, II = power, III = swallowing, IV = recovery. The vertical bars in A represent the standard deviation in each interval.

of selected film sequences were measured frame by frame, and recalculated to a reference grid defined by two markers on the neurocranium. Movement analysis involved (1) the construction of time-displacement graphs, which allows precise determination of the direction, amplitude and frequency of the pharyngeal jaw movements, (2) calculation of the angles between the toothplanes and the neurocranium (Fig. 1), which allows detection of pharyngeal jaw rotations around a transversal axis, and (3) calculation of mean movement cycles (Figs 2, 3). To facilitate the interpretation of the time-displacement graphs, a computer program was made to design successive pharyngeal jaw contours relative to the stationary neurocranium outline (Fig. 3). The methods of movement analysis are described in detail in CLAES and DE VREE (1991).

RESULTS

The pharyngeal jaw apparatus of *Haplochromis burtoni* closely resembles that of the related species *H. elegans*. Detailed descriptions of the pharyngeal jaw apparatus in *H. elegans* are given by BAREL *et al.* (1976) and ANKER (1978, 1989).

Pharyngeal food processing is effected by cyclic movements of the pharyngeal jaws. The protracted upper jaw position is chosen arbitrarily as the starting point of each cycle and practically corresponds with the most retracted position of the lower pharyngeal jaw (Figs 2 and 3). Subdivision of each cycle is based on the different movements of the upper and lower pharyngeal jaws. In this way, three major phases are distinguished during upper jaw retraction (preparatory, power and swallowing), while the period of upper jaw protraction is regarded as a separate phase (recovery).

The lower pharyngeal jaw is kept almost immobile during the preparatory phase, while the upper pharyngeal jaws are slightly retracted (Figs 2 and 3). The angle between the toothplanes of the upper pharyngeal jaws and the neurocranium (Angle 1 in Figs 1 and 2) increases, indicating a clockwise upper jaw rotation (head tip to the right in Figs 1 and 3), and resulting in tooth-food contact in preparation for the power phase.

During the power phase, the lower pharyngeal jaw moves anterodorsally and subsequently anteroventrally (Fig. 2) ; distinction between these movements allows further subdivision into compression and shearing phases. Upper jaw retraction and rotation decreases during the compression phase, while the lower pharyngeal jaw is lifted and simultaneously rotated clockwise (increase of angle 2 in Fig. 2) ; lower jaw protraction is limited during this phase (Figs 2 and 3 : compression). The shearing phase is characterized by strong lower jaw depression and protraction, while the upper pharyngeal jaws are further retracted and rotated clockwise (Figs 2 and 3 : shear). At the end of the power phase, the lower pharyngeal jaw is fully protracted and reaches its ventralmost position.

The lower pharyngeal jaw is retracted during the swallowing phase while kept in its ventralmost position. Although the upper pharyngeal jaws are only slightly retracted during the swallowing phase, they are further rotated caudodorsally,

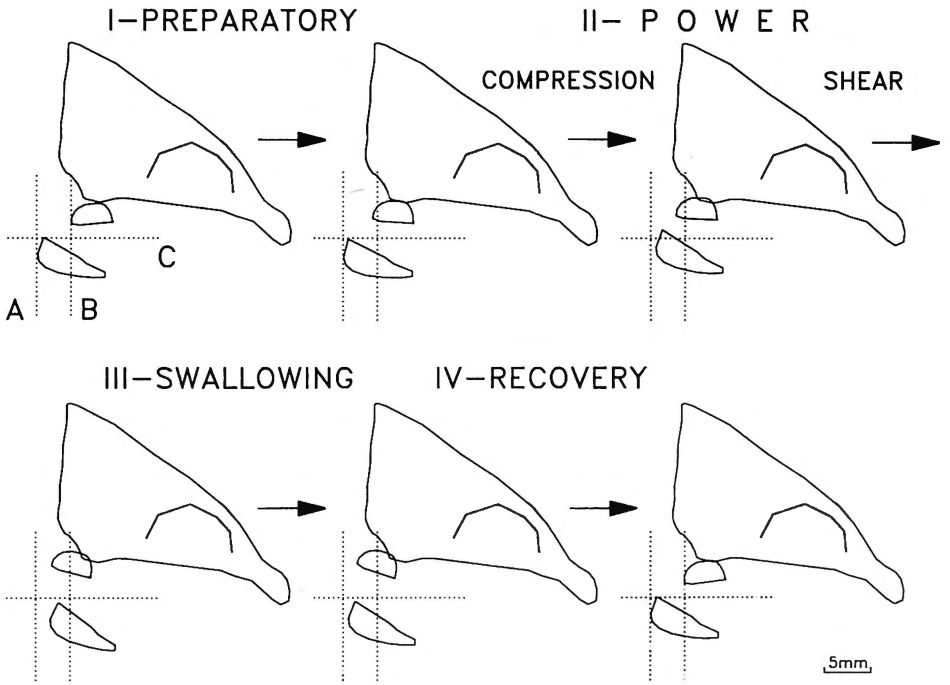

Fig. 3. — Outlines of the pharyngeal jaws and the neurocranium for the different phases, based on the same mean cycle as depicted in Fig. 2. Reference lines A,B and C indicate the position of the jaws at the start of the cycle. A = most posterior lower jaw position, B = most anterior upper jaw position, C = reference for dorsoventral lower jaw displacements.

enabling deglutition of the posteriormost food particles. The angle between the toothplanes of the upper and lower pharyngeal jaws (angle 3 in Figs 1 and 2) decreases to its minimal value as a result of the caudodorsal (clockwise) upper jaw rotation and a slight anticlockwise lower jaw rotation. Consequently, the toothplanes of the upper and lower pharyngeal jaws lie almost parallel to each other (Fig. 3).

Finally, the pharyngeal jaws move to their initial positions during the recovery phase : the upper pharyngeal jaws are protracted and rotated anticlockwise, the lower pharyngeal jaw is further retracted and moves sligtly upward (Figs 2 and 3).

Although the amplitudes of the displacements and the durations of the different phases may vary within a masticatory sequence, this kinematic pattern is basically stereotyped. The cycle duration ranges between 440 and 580 ms (N = 11, mean = 497 ms).

DISCUSSION

The first description of the pharyngeal biting mechanism in cichlids was based on experiments with *Haplochromis burtoni* (LIEM, 1973), feeding on *Gammarus* species. This experimental study emphasized muscle activities (electromyography) during pharyngeal food processing, whereas the movements of the pharyngeal jaws were deduced from successive X-ray pictures. LIEM (1973) described a triphasic movement cycle, in which the upper and lower pharyngeal jaws show synchronous anteroposterior displacements. During the first phase (phase « 1a », LIEM, 1973), the pharyngeal jaws are closed during protraction, resulting in the application of force on the food. During the second phase (phase « 1b », LIEM, 1973) the pharyngeal jaws are strongly retracted. The pharyngeal jaws are protracted and abducted during the third phase (phase « 2 », LIEM, 1973). The transition between the end of « phase 2 » and the onset of « phase 1a » (depicted in LIEM, 1973, Fig. 8) is not considered in the description of the movement cycle, although it should be regarded as a fourth phase.

A different triphasic movement pattern was described by LIEM (1978) for piscivourous cichlid fishes, and, in a later paper (LIEM, 1986, p. 321) also for *Haplochromis burtoni*. The movement cycle described in these papers consists of two power strokes separated by a transitional stroke. During the first power stroke (also called « shearing » in LIEM, 1986) the upper and lower pharyngeal jaws are protracted while approaching each other. The transitional stroke is sligthly retrusive. During the second power stroke (« crushing » in LIEM, 1986), the pharyngeal jaws close a second time during their retraction. Thus, according to LIEM (1978, 1986), cichlids masticate food during two « power strokes », separated by a « transitional stroke », while the upper and lower pharyngeal jaws show simultaneous protraction and retraction.

The results in the present paper clearly demonstrate that *Haplochromis burtoni* does not show either one of these triphasic movement patterns when feeding on crickets and earthworms. Based on quantitative cineradiography (i.e. measurement of bone displacements in a standardized reference grid), each movement cycle is subdivided into four main phases : a preparatory phase (upper jaw retraction, nearly stationary lower jaw), a power phase (upper jaw retraction, lower jaw lifting and protraction), a swallowing phase (upper and lower jaw retraction) and a recovery phase (upper jaw protraction, lower jaw retraction). Throughout most of the movement cycle (power phase, recovery phase), the upper and lower pharyngeal jaws thus move in opposite anteroposterior directions ; the moment of maximal upper jaw protraction corresponds with that of the most retracted lower jaw location. The morphology and orientation of the pharyngeal teeth suggest that this movement pattern produces an effective and continuous shear stress during the power phase. This movement pattern agrees with that of *Oreochromis niloticus* (CLAES and DE VREE, 1989, 1991).

It is unlikely that the differences between our results and the kinematic patterns described by LIEM (1973, 1978, 1986) are due to differences of food-types, since the movement pattern in *Haplochromis burtoni* is consistent when using earthworms

and crickets. A detailed study of *Oreochromis niloticus* (CLAES and DE VREE, 1991) revealed a significant influence of food type on the durations of the different phases, but not on the basic movement pattern (relative directions of the upper and lower pharyngeal jaw displacements). It is more likely that deduction of movements from muscle activities does not allow a detailed description of the kinematics, increasing the chances of misinterpretations of valuable experimental results. Anyway, our results show that the movement pattern described by LIEM and GREENWOOD (1981) should not be generalized for the whole family. In expectation of further verifying research, the triphasic pattern in piscivorous cichlids (LIEM, 1978) should be regarded as exceptional within the cichlid family.

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IMMUNOREACTIVITY OF *BUFO MARINUS* HEART FOR ATRIAL NATRIURETIC FACTOR

by

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SUMMARY

This communication shows that the toad *Bufo marinus* L. heart contains immunoreactive-atrial natriuretic factor (IR-ANF) which is largely present in the atrial myofibers and in several layers of cardiomyocytes of the ventricles. Atrial myocytes of the truncus arteriosus wall also show IR-ANF content. Functional aspects of *Bufo marinus* body fluid homeostasis related to these observations are discussed.

Key words : heart, Amphibian, atrial natriuretic factor, immunohistochemistry, body water regulation.

INTRODUCTION

In 1956, KISCH showed that the sarcoplasm of atrial myocytes contains dense cored vesicles or granules. Since these granules were exclusively observed in the cardiac myofibers, they were named « specific » granules (JAMIESON and PALADE, 1964). Because the number of granules was altered with changes in bodily water-salt balance (BENCOSME and BERGER, 1971), it was suggested that an endocrine factor was contained in the heart and secreted upon demand from the heart to facilitate excretory functions (DE BOLD, 1979, 1989 ; DE BOLD *et al.*, 1981). Biochemical analysis of the atrial granules (FLYNN and DAVIES, 1983) demonstrated that these physiological effects were caused by a polypeptide which was contained within these atrial granules. A family of peptides was isolated and sequenced, and among these one was named atrial natriuretic factor [ANF] (FLYNN and DAVIES, 1983). ANF is produced as a portion of a larger precursor polypeptide molecule (126 amino acids in length or proANF) which represents the major storage form of the peptide inside the secretory granules (FLYNN *et al.*, 1983 ; GELLER *et al.*, 1984). In several reports we have shown that the cardiac endothelial linings of the endocardium, the blood vessels, and the epicardium were structural barriers which could control and

regulate the transport of ANF towards the circulation (GILLOTEAUX and LINZ, 1990 ; GILLOTEAUX *et al.*, 1988, 1991). We have also suggested that these endothelia could also be important routes to activate proANF before its release into the circulation as ANF (1-28). In addition, based on our observations and other reports, we have proposed that the integrity of such endothelia was critical to specifically activate ANF before or at the site of release into the circulation (GILLOTEAUX, 1990 ; GILLOTEAUX *et al.*, 1991).

Comparative developmental, biochemical, and immunohistochemical studies originating from lower Vertebrates are still scarce even though they could provide precious information about the physiology of the cardiac-kidney-adrenal cortex axis and other organs associated with sodium and water excretion (skin, gills, etc.) (FORSSMANN *et al.*, 1989). New information obtained from comparative work could also shed light into morpho-functional characteristics of this cardiac peptide in all Vertebrates. This would be most valuable from studies investigating species adapted to peculiar or specialized ecological niches. More morphological and molecular information is currently being collected from a range of several species (fishes, amphibians) in our laboratory. Some of the preliminary observations of ANF immunolocalizations detected in cardiac tissues of the toad are described in this report.

MATERIAL AND METHODS

Organ used : Four hearts from male toads (*Bufo marinus* L.), kept at constant temperature (8 ° C) under running water with a 12-hr light, 12-hr dark cycle in the animal care facility of the Physiology laboratory of the Free University of Brussels, were obtained from the Laboratory of Physiology of the Free University of Brussels during sabbatical leave in the Neuropathology and Electron Microscopy Laboratories in the Erasme-Anderlecht Campus.

LM immunohistochemistry : Hearts were fixed either by 4 % buffered formaldehyde (0.1 *M* phosphate) or by Bouin-Hollande sublimated-trichloracetic mixture for a maximum of 5 hours by immersion before being embedded in paraffin. Samples fixed in the Bouin-Hollande fixative were washed for 3 hours in running tap water before dehydration and embedding in paraffin. Both techniques gave identical results but a denser immunoreactivity was observed after Bouin-Hollande fixation. Seven-µm thick sections were cut and,following deparaffinization, STERBERGER's (1979) indirect immunohistochemical method, using peroxidase-antiperoxidase (P.A.P.), was employed to visualize ANF immunoreactivity sites of the rabbit anti-ANF serum # 10-5 antibodies in serial sections of toad hearts following the procedure previously described in GILLOTEAUX *et al.* (1991). Immunostained patterns were then observed and photographed in a Zeiss photomicroscope. Alternate sections were stained by hematoxylin-eosin (H & E). Specificity control tests included preabsorption of the ANF antiserum with somatostatin, CCK, or NPY did not reduce the intensity of immunostaining ; omission of the primary antiserum, incubation with preimmune serum (1/1000), and

preabsorption of the primary antibody with 10^{-6} *M* synthetic atriopeptin III(5-25) or α-human-ANF(1-28) [Bachem AG. and Peninsula Co.] gave negative results.

ANF antiserum # 10-5 : this anti-ANF serum was a gift from Dr L. Jennes (Wright State University, Dayton, OH). It was obtained by immunization of a rabbit with synthetic atrial natriuretic peptide (rat 5-28) which was coupled to keyhole limpet hemocyanin via 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide-HCl (JENNES and STUMPF, 1983). The antiserum recognizes atrial natriuretic peptide (5-15), ANF(1-28), and beta-rANF(17-48). These data show that the antiserum should bind to free active ANF and to ANF as a portion of the precursor molecule. Since the antibody does not recognize the ANF fragment (18-28) it is suggested that (a) an intact cystine bridge is required for binding or (b) the portion of the peptide in close vicinity with the N-terminus is the site which is recognized by the antibody.

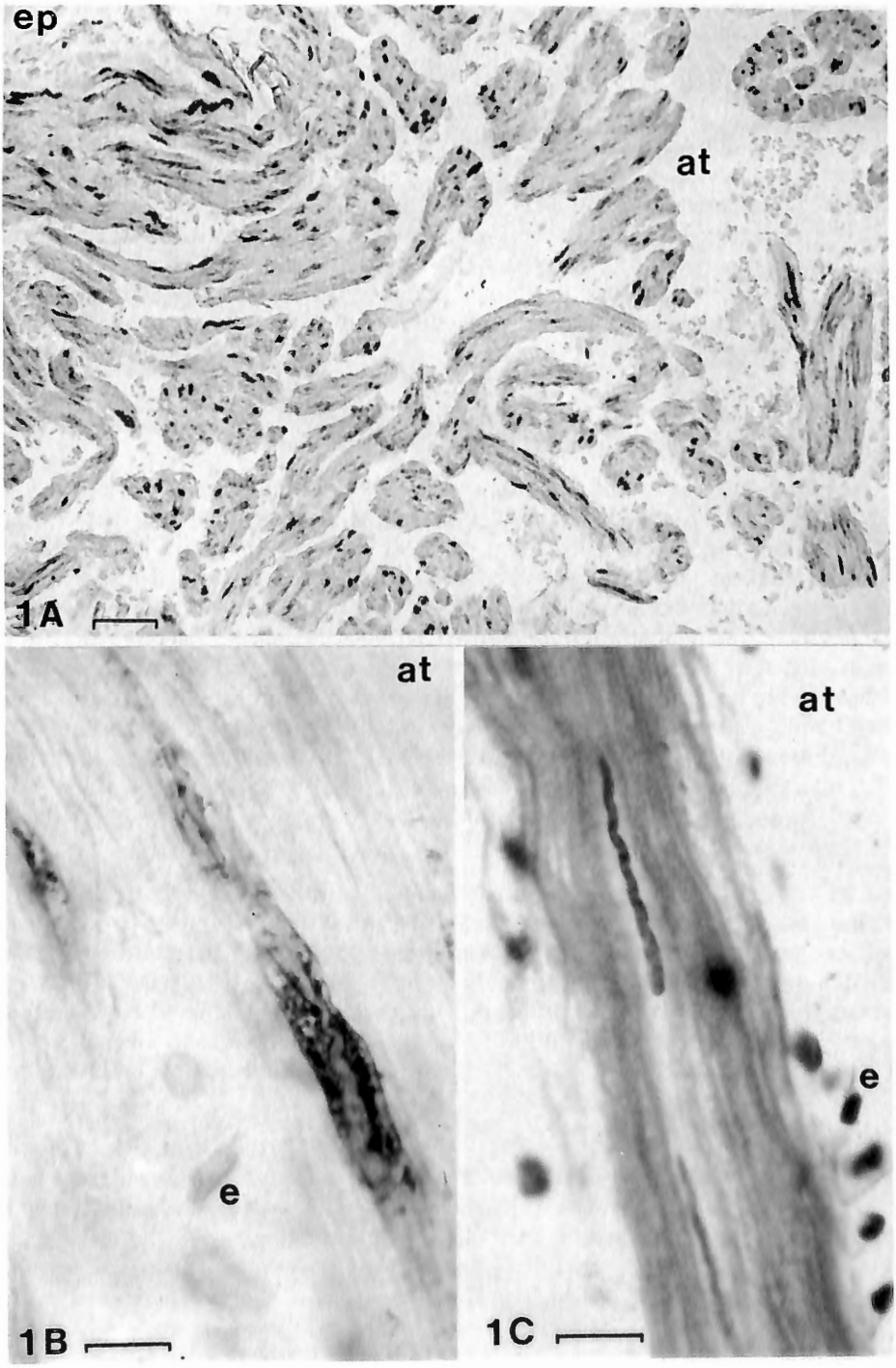
RESULTS

As in mammalian hearts, the atrial walls of this amphibian are less thick (0.15-0.58 mm) than the ventricular wall (0.28-0.83 mm). A loosely, delicate, trabeculated network of atrial myocytes bundles ranging from 25 to 150 μm in diameter originate from the thin atrial walls and two distinct chambers are clearly separated. The right atrial wall is almost twice as large as the left one. The ventricle extends 15 mm in height from the atrial-ventricular junction and is about 13 mm in its widest diameter. It shows only a small open cavity (less than half the radius of the ventricle) in its upper region. This muscular chamber, although made of trabeculated muscular bundles, appears more compact than both atrial chambers and communicates with the open cavity via a large number of small fissures between trabeculated strands of myocardial bundles. A narrow endocardial lining of 5 to 12 μm covers the cardiac luminal surfaces and an epicardial layer, 25-100 μm in thickness, covers the myocardium (Pl. I, 1A and Pl. II, 2).

In this study the respective measurement of atrial versus ventricular myocyte lengths and width are not attempted as our attention focused on the description of immunostaining patterns. The atrial cardiomyocytes attain 5-16 μm in width. They are spindle-shaped, branching, and are often binucleated (Figs 1B-C). The ventricular myocytes are typically branching and their diameter ranges between 12 and 20 μm. After H & E staining, both atrial and ventricular myocytes show a narrow, pale perinuclear zone suggestive for the locations of the RER, Golgi apparatus, and ANF-containing granules.

As expected, the immunostaining patterns of the atrial (Pl. I, 1B) and ventricular (Pl. II, 2) myocytes are localized in the same aforementioned perinuclear cell regions. All the myocytes of the atrial walls show an intense and positive granularity in a 5-8 μm thick perinuclear sarcoplasmic region which corresponded to the most medial zone of each myofiber (PL. I, 1B). Following a careful examination of the immunohistochemical localizations, immunostained structures can be detected throughout the sarcoplasm of the muscle fibers, but with a decreased

PLATE I



intensity from the perinuclear region toward the fiber peripheral regions (Pl. I, 1B compared with Pl. I, 1C).

Interestingly enough, a subepicardial layer of 8-14 immunostained cardiomyocytes is detected in the ventricle. Furthermore, scattered stained myocytes can also be observed within deeper, thick trabeculae of the ventricular wall (Pl. II 2 where immunoreactive sites are exemplified by arrows). ANF-immunoreactivity appears restricted to the perinuclear sarcoplasm, since a dispersed and only faint staining is shown in the more distal regions of the ventricular myocytes (Pl. II, 2 inset). Finally, a narrow layer of immunostained myocytes is observed in cells adjacent to the endothelium of the lumen of the truncus arteriosus. These myocytes measure not more than 7 µm in diameter (Pl. II, 3). Immunoreactive sites for ANF products in the toad hearts are summarized in Fig. 1.

DISCUSSION

Comparisons between immunoreactive staining observations and hematoxylin-eosin stained sections show that all the toad myocytes detected show a similar ANF-IR staining localization as the mammalian myocytes where similar rabbit antibodies raised against synthetic rat ANF (Arg 101-Tyr 126) produced immunochemical staining in all the toad atrial myocytes. It is not surprising to find that ANF-like peptides can be detected in the walls of the amphibian ventricle since a small number of authors (CANTIN *et al.*, 1987 ; CHAPEAU *et al.*, 1985 ; NETCHITAILO *et al.*, 1986, 1988 ; TRILLO *et al.*, 1986) have already reported comparable observations in frog heart, but without illustrating their descriptions about the ventricular wall.

An ultrastructural survey of the cardiac tissues of this type of vertebrate is not described in this report because tissues were given after they were fixed by Bouin or formaldehyde. However, using the same antiserum and immunoelectron gold labeling technique used with success in other investigations, it was demonstrated that the atrial granules in the Syrian hamster (GILLOTEAUX *et al.*, 1991 ; GILLOTEAUX and LINZ, 1990) and in the carp (*Cyprinus carpio* L.) (GILLOTEAUX and XU, in preparation) contain ANF-like product. Consequently, it is possible to

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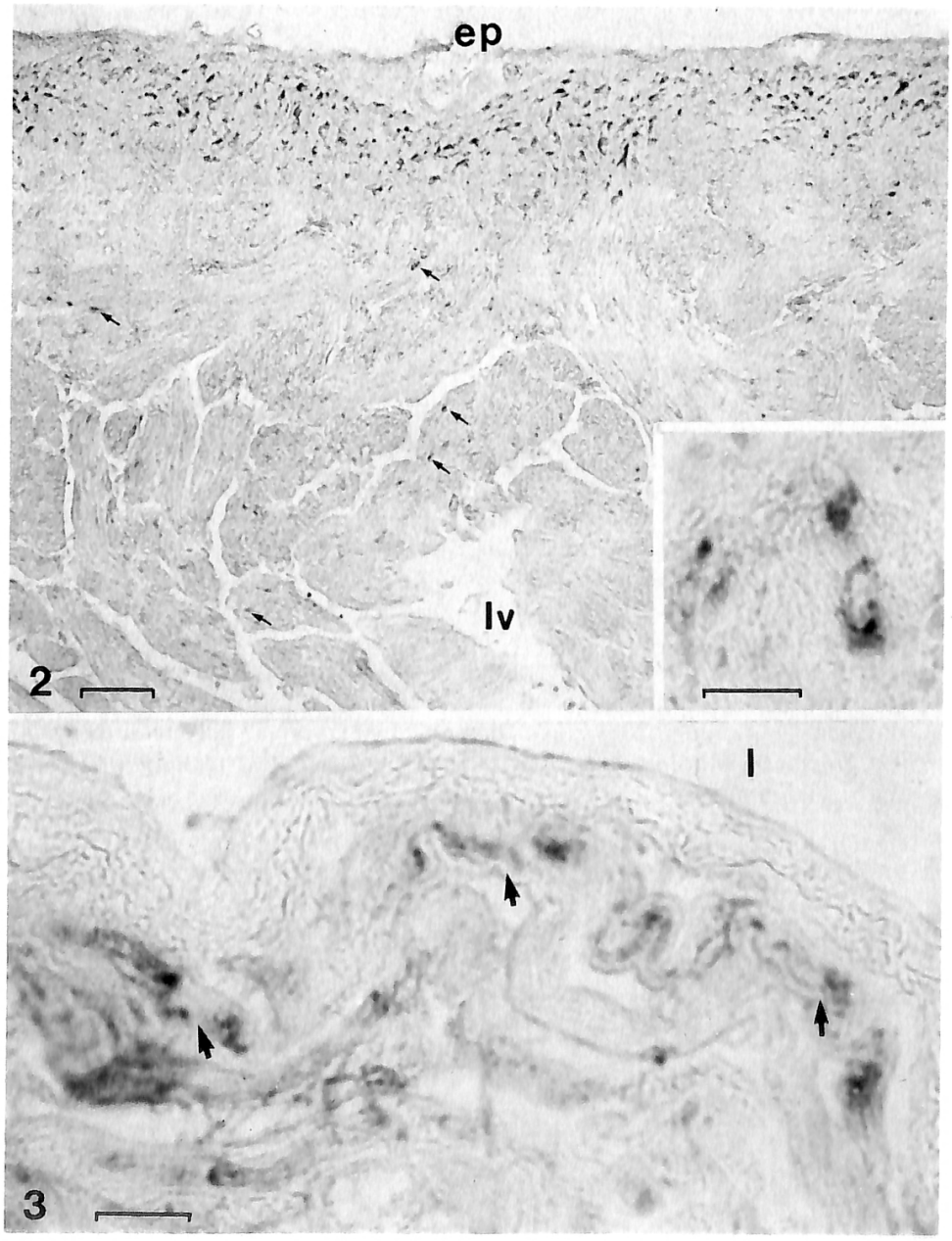
PLATE I

1 A-C - *Bufo marinus* right atrium : at : atrium chamber ; e :erythrocytes ; ep : epicardial layer. In A : Region of the atrial wall and trabeculae which shows dense ANF immunoreactivity localized in the perinuclear regions of each atrial myocyte. An enlarged myocyte is illustrated in B. PAP indirect method using anti-ANF # 10-5 diluted 1 :4000 [v/v], no counterstain. In C : Detailed view of nuclear region of an atrial myocyte depicting a pale, circumnuclear zone following hematoxylin-eosin stain. Scales : in A is 100 µm ; in B and C is 10 µm.

PLATE II



suggest that the immunostained areas correspond to the presence of sarcoplasmic granules containing a peptide of close composition and stereochemistry to ANF. The positive cross-reaction allows the identification of the sites of production and storage of ANF-like compound probably contained in atrial and ventricular granules in the toad. The cross-reactivity of similar ANF antiserum with the frog atrial tissues indicates that an ANF-like compound or a molecular precursor is well conserved among all vertebrates (GILLES *et al.*, 1990 ; KIM *et al.*, 1989 ; LAZURE *et al.*, 1988 ; SAKATA *et al.*, 1988).

Though the immunoreactivity, at first, appears weaker in the ventricular wall, the large number of myocytes and the large size of the ventricle suggest that there is likely to be more ANF produced in the ventricle than the atria. Meanwhile, it is not yet demonstrated whether the mode of secretion is continuous or regulated in a similar way to that which BLOCH *et al.* (1986) showed in the mammalian ventricular tissues. There it was found that the content is released continuously while the atrial myocytes secrete ANF, following a regulated mode of transport. ANF is also largely expressed in the ventricle in human hypertrophic cardiomyopathies (KAWAMURA *et al.*, 1991), during late fetal development, and in the perinatal age (RASCHER *et al.*, 1987 ; SMITH *et al.*, 1989). There, ANF is distributed in the same pattern as detected in these lower vertebrates and provides a high plasma level of ANF. It is appropriate to speculate that cardiac stress or specific myopathic defects can favor the production and release in the circulation of large amounts of ANF needed to facilitate the vasodilation of blood vessels and to compensate the compromised cardiac output. During pregnancy, the mammalian fetus lives in an uterine cavity provided with an unlimited amount of fluid whereas in the first few days following delivery an important and necessary diuresis and natriuresis occur. After a week of age the neonate heart decreases its ANF production (SMITH *et al.*, 1989 ; TULASSAY, 1988). This phenomenon is certainly a response to potential dehydration and to maintain survival in a « dry » environment.

The functional significance of a larger number of ANF sites of production and release in the toad could relate to the fact that this toad, like other Amphibians, was kept in an aquarium instead of a terrarium ; in the aquarium, the abundant water intake needs to be excreted continuously and diluted urine is formed, as in

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PLATE II

2 - Ventricular wall demonstrating ANF immunohistochemical staining in a wide subepicardial layer of myocytes and in interspersed myocytes of the myocardium (examples are arrowed). e : epicardial layer ; lv : left ventricle chamber. Scale is 100 µm. Insert shows oblique sections of three immunostained myocytes ; scale is 10 µm. PAP indirect method anti-ANF ▷10-5 diluted 1 :4000 [v :v], no counterstain.

3 - Subluminal region of truncus arteriosus wall where ANF immunostaining is detected in a small number of atrial myocytes (examples are arrowed) ; l : lumen. Scale is 10 µm.

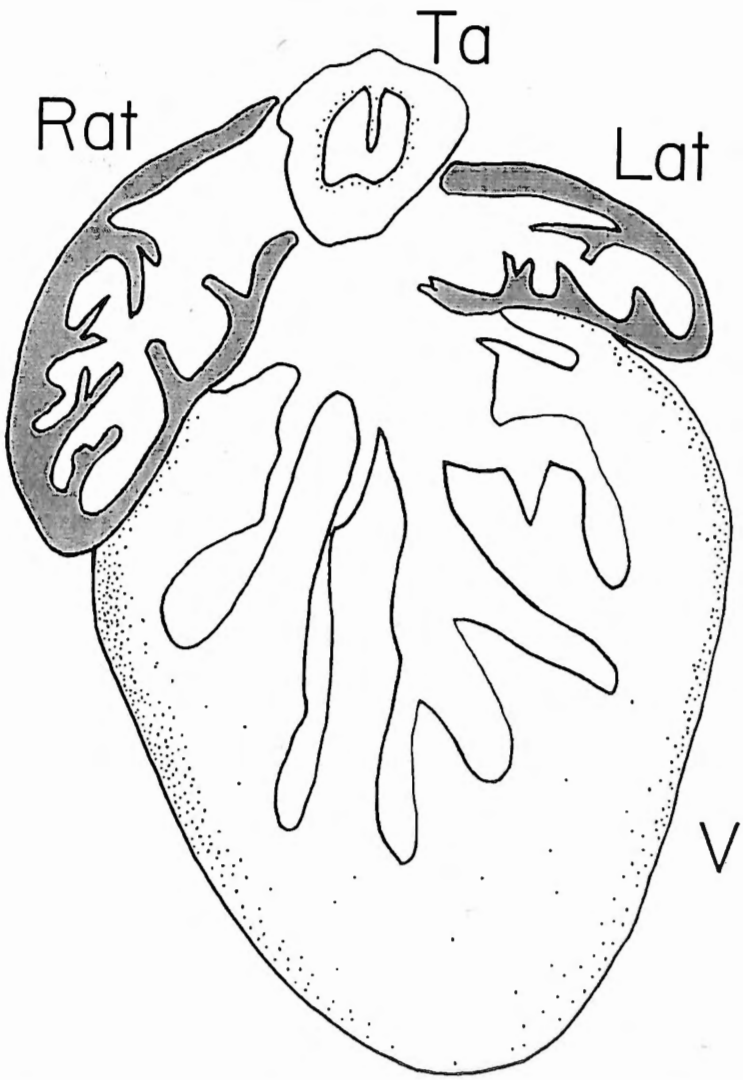

Fig. 1. — Diagrammatic representation of ANF-immunostaining pattern in *Bufo marinus* heart. Atrial cells contain ANF and are depicted by shading of the atria. The ventricular wall immunoreactivity is less intense and its distribution is represented by a stippled pattern. Lat : left atrium, Rat : right atrium, Ta : truncus arteriosus, V : ventricle.

frogs. Anurans are able to osmoregulate between hypo- to isotonicity whereas *Bufo marinus* is adapted as osmoconformer (SHOEMAKER, 1977). These adaptations meet large changes in environmental salinity and are probably related to the production

and secretion of large quantities of ANF, since a large supplemental sodium and water intake is continuously provided through the skin (DEYRUP, 1964 ; DUELLMAN and TRUEB, 1986). Skin and kidneys are the main sites for water regulation in an amphibian, especially in this terrestrial anuran. As water is 70-80 % of the total body weight of most amphibians, a critical function of the kidneys is to continually excrete an hypotonic urine, even in the case of strongly dehydrated toads (LEAF *et al.*, 1958). However, *B. marinus*, a brakish-adapted amphibian, would contain less IR-ANF in the heart ventricles than the frog, because the maintenance of their osmoregulatory homeostatic mechanisms is less critical than those of amphibians whose habitat and ecological niche is quasi restricted to freshwater (GILLES *et al.*, 1990). This assumption is being investigated in our laboratory. While altering the external milieu, we are measuring ANF mRNA production in the three cardiac regions where ANF immunoreactivity is described in this report.

Finally, the water-electrolyte balance in *Bufo* is dependent on blood circulation. The lymphatic system is an important, often neglected, component of an extensive system for electrolyte and metabolite exchanges in these vertebrates (CARTER, 1979). Lymph heart contractions and lymph flow can be modulated and controlled by aldosterone, produced in abundant interrenal tissues (CRABBÉ and DE WEER, 1964 ; ZEIDEL, 1990). In addition, it is possible that these supplemental regions of production of ANF, antagonistic to mineralocorticoids, would certainly grant that additional, production sites of ANF-like compounds originate from these poorly-studied lymphatic hearts. Their secretion (probably made of ANF-like compounds) could counterbalance the large amounts of antidiuretic arginine-vasotocin and aldosterone-secreting structures (RUMYANTSEV and KRYLOVA, 1990 ; SHOEMAKER, 1977).

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THE MANY MEANINGS OF GREAT TIT SONG

by

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SUMMARY

Studies on Great Tit song investigated song characteristics mainly influenced by song learning. Great Tit song provides information on the origin of birds (e.g. song dialects), and on the habitat type in which the birds live. Experimental studies yielded a series of hypotheses about the functional meaning of song type structure and song repertoires. Detailed studies of individual differences in great tit singing performance, that are summarised here, revealed that variation in great tit song is not only caused by learning ability and learning opportunities, but could also be influenced by constraints in singing ability. These studies showed that great tit song reflects male quality.

Keywords : Song ; *Parus major* ; function ; review.

INTRODUCTION

Since the mid-seventies about 30 papers have been published on various aspects of great tit (*Parus major* L.) song in order to search for answers to the very complicated question why birds in general, and great tits in particular, would spend so much time and energy in singing. In this paper we review how the problem has been studied so far, with particular emphasis on the work carried out in Antwerp, and make some suggestions for future studies.

Male great tits sing a very simple song, which they repeat a great many times. The smallest unit of great tit song is a group of one to more than five different notes that is called a phrase. A phrase is rapidly repeated in a stereotyped way in a short burst of song that is called a strophe. A strophe may consist of one to more than 20 phrases, and lasts normally between one and five seconds. Great tits sing a strophe. Then they pause a few seconds, and start all over again. If they sing for several minutes they will probably change to a different version of the species-specific song (i.e. another song type), etc. (Fig. 1) (MCGREGOR and KREBS, 1982 ; LAMBRECHTS and DHONDT, 1988a).

VARIATION IN SONG TYPES AND REPERTOIRES

The first song studies in the great tit made detailed descriptions of what kind of song types are found in different populations, how song types are distributed within populations, and how many song types individuals sing. Different song types show differences in phrase structure (number of notes per phrase, note length, note frequency,...) that can easily be recognized on sound spectrograms (Fig. 1). Although in a given study population one may hear 30-50 different song types, each individual male only sings between one and six, rarely more different song types. Each individual has a unique song repertoire composition (i.e. the song types that constitute the repertoire). Birds learn most of their song types before their first breeding season. The composition of the repertoire is not random in that birds are more likely to learn (copy) song types from territorial neighbours. Therefore, neighbouring males are more likely to sing the same song type than other birds, and song types may differ between populations. Habitat type has an effect on song type structure, whereby in more open park-like habitats complex song types (i.e. with more notes per phrase) are more common (EYCKERMAN, 1979 ; HUNTER and KREBS, 1979 ; MCGREGOR and KREBS, 1982, 1989). Therefore, song type structure and the repertoire composition gives information about where an individual lives.

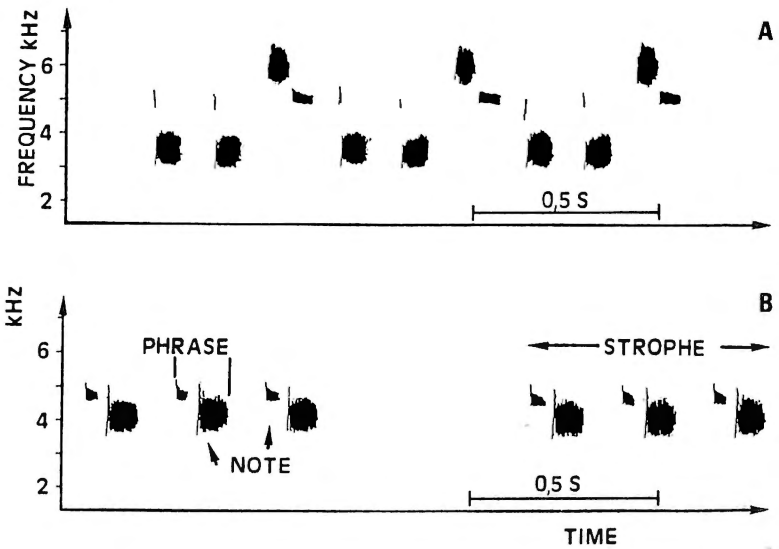

Fig. 1. — Examples of sonagrams (frequency against time diagram) of two songtypes of a Great Tit. Notes make up a phrase. Phrases are repeated in a strophe. Strophes of the same song type are repeated in a bout.

In A a complex four-note songtype is repeated three times.

In B two three-phrase strophes of a two-note songtype are shown, separated by an interstrophe pause. Drift can both occur within a strophe, by increases in interphrase pauses, as between strophes by increases in interstrophe pauses and reduction of strophe length.

Since song is mainly produced during the pre-breeding and breeding season one can reasonably assume it has to do with reproduction (e.g. EYCKERMAN 1979 ; MACE 1987), i.e. with territorial defence and with mate attraction or stimulation. This hypothesis is supported by a number of experimental studies. Great tits start to sing on their territory when they are presented with playback of the conspecific song (e.g. DHONDT, 1966). In territories with song intrusion is slower than in territories without song (KREBS, 1976a, 1977b). Finally, territorial males that lost their mate sing much more frequently than mated males (KREBS *et al.*, 1981a), and song stimulates sexual behaviour in females (BAKER *et al.*, 1986, 1987).

The detailed descriptive studies of song types and repertoires allowed investigators to ask very specific questions about the functional meaning of variation in great tit song : Why is great tit song simple in comparison with other species ? ; Why do great tits have a song repertoire ? ; Are some song types more effective in territorial defence than other song types ? ... Although in some species song types that are used in mate attraction differ from song types that are used in territorial defence in the great tit there is no evidence that different song types have a different functional meaning (KREBS, 1977a). The simple, stereotyped songs of great tits may facilitate individual recognition which could be important in territorial defence or in female choice. Indeed, male great tits respond differently to familiar song types (i.e. song types of neighbours) than to unfamiliar song types in a playback experiment. Therefore, great tits may use song type structure in neighbour-stranger recognition (FALLS *et al.*, 1982), even if the birds do not sing the song types themselves (MCGREGOR and AVERY, 1986). Also, a female is more likely to mate with a male that sings song types different from that of her father but similar to the song types he sings (MCGREGOR and KREBS, 1984a). BAKER *et al.* (1987) showed experimentally that local song types sexually stimulate females more than distant song types, which suggests that song type structure could be used in mate choice.

REPERTOIRES AND TERRITORY DEFENCE

KREBS *et al.* (1978) showed experimentally that song repertoires are more effective in keeping out intruders than single song types. MCGREGOR *et al.* (1981) found that males with larger song repertoires survive better, have a higher breeding success and a greater lifetime reproductive success. They suggested therefore that males with larger repertoires obtain better territories because they would be more successful in territorial defence. Several hypotheses have been proposed why song repertoires are more successful in male-male competition. According to the anti-habituation hypothesis the signal would lose strength unless the song types used are regularly changed (KREBS, 1976b). The Beau-Geste hypothesis assumes that song repertoires would mislead potential settlers into believing that the woodland is more densely occupied than it really is (KREBS, 1977a). A third hypothesis is that song repertoires allow birds to match song types with different individuals. In territorial conflicts males often match, which is the phenomenon that they select that song type in their repertoire that most closely resembles that of the opponent. Song type

matching would improve territorial defence because it would be a mechanism (1) to direct a signal to a singing opponent, (2) to give information about the willingness to attack of the occupier that responds with the same song type (KREBS *et al.*, 1981b), or (3) to give information about the distance between singing birds (MCGREGOR and KREBS, 1984b). Finally females that mate with a male singing many song types could receive a reproductive advantage because song repertoire size reflects male or territory quality. This may explain why larger song repertoires sexually stimulate females more effectively than smaller ones (BAKER *et al.*, 1986).

HOW DO GREAT TITS SING ?

The development of song of an individual can be divided into an input phase in which song types that are heard in the population are stored in the brain (memorized), and an output phase in which the song types that were learned are produced using a set of respiratory and syringeal muscles (KROODSMA and MILLER, 1982). Most of the song studies in the great tit (and in other species) were about those song features that are influenced by the phenomenon of song learning, such as the composition of song repertoires and the distribution of song types in populations : thus those features that describe what great tits sing. These studies were very successful in that they showed that song is for territorial defence and for mate attraction or stimulation, and that birds with large song repertoires were better at that.

In Antwerp we have less asked the question 'what do great tits sing' than 'how do they sing it ?'. As explained before great tits repeat in a very stereotyped fashion a particular song type, pause, then sing again. If one carefully listens to a strophe one can hear, in many strophes of many birds, drift. Drift is a slowing down of the rhythm within a strophe, caused by an increase in the pauses between notes and between phrases (LAMBRECHTS and DHONDT, 1987). Because a strophe only lasts a few seconds, it seems unlikely that this drift within a strophe would be the result of a change in motivation to sing, the more so, because a few seconds later the same bird will begin its next strophe again with no drift. If drift is not caused by a change in motivation it could be the result of, for instance, physiological or respiratory problems for the bird. We hypothesized therefore that drift might reflect a male's singing ability. Males able to sing well would show no drift, whereas males not able to sing well would show pronounced drift.

Our hypothesis about the causation of drift was supported by detailed descriptions of drift in different individuals (LAMBRECHTS and DHONDT, 1987). Comparing strophes with the same number of phrases some birds showed much more drift than others. In strophes longer than the bird's average strophe length there was pronounced drift, but in strophes shorter than the bird's average strophe length there was no or little drift. If drift would reflect a shortterm decrease in motivation to sing we expected to find the opposite, i.e. more drift when a bird produces strophes that are shorter than its average strophe length.

As measuring drift is very time consuming, and drift and strophe length are correlated, and as we needed to measure many song bouts from many birds, we used strophe length as a measure of male singing ability, rather than and instead of drift. As with drift we found that strophe length varies considerably between males. It also varied between song types and with season, although all measures of strophe length of a male were intercorrelated. In some males, with a high singing ability, the average strophe length of the repertoire was more than 10 phrases per strophe while in other males the average strophe length was not more than five phrases per strophe (LAMBRECHTS and DHONDT, 1987).

SINGING ABILITY AND FITNESS COMPONENTS

The next logical step was to investigate how far singing ability (defined by drift or by strophe length) reflected male quality, i.e. was related to fitness components. Social dominance in winter influences survival (KIKKAWA, 1980 ; ARCESE and SMITH, 1985 ; DE LAET, 1985). We therefore studied winter dominance and singing ability in the same males and found that dominant birds in winter were better singers in spring. This higher survival of the better singers was particularly clear between the first and the second breeding season, and better singers lived longer (LAMBRECHTS and DHONDT, 1986).

Social dominance, in many species, is related to early settlement, whereby individuals that settle first are more dominant (DHONDT, 1971a ; KREBS, 1982 ; BRAWN and SAMSON, 1983 ; DE LAET, 1985 ; HOGSTAD, 1987 ; NILSSON and SMITH, 1988). TEUNEN (1987) found, after a sudden massive mortality of the resident males in early January, that there was a clear correlation between date of settlement and strophe length : good singers settled significantly more rapidly than poor singers.

The second factor which influences fitness is the number of recruits produced in an individual's lifetime. We found that better singers were not more successful at recruiting offspring in a single breeding season, but did have a higher lifetime reproductive success (more recruits) because they bred in more different seasons.

A male's singing ability, as measured by strophe length and drift, is therefore a reliable measure of male quality (LAMBRECHTS and DHONDT, 1986). Male quality can thus be assessed easily just by listening to a singing bird.

SINGING ABILITY AND TERRITORY QUALITY

As song is used in territorial defence and female attraction, and song reflects male quality, we expected that birds with longer strophes and less drift would be more successful in male-male competition and in female attraction.

At the behavioural level strophe length seems to be important in male-male interactions. Thus when males were confronted with playback of long strophes (LAMBRECHTS and DHONDT, 1987) individuals naturally singing long strophes increased their strophe length in response to playback, while males with very short strophes started to sing even shorter strophes. This suggest that strophe length is

used in territorial defence and that long strophes could inhibit territorial (aggressive) behaviour in some males. However, further experiments (cf. KREBS *et al.*, 1978) are needed to examine if longer strophes are more successful in keeping out intruders than shorter strophes.

Another suggestion why strophe length and drift could be important in male-male conflicts follows from the observation that in escalated conflicts, when the chance of a physical fight is high, males match. Matching is the phenomenon whereby males involved in a song duel countersing with a song type that closely resembles that of the challenger (KREBS *et al.*, 1981). Theory predicts that before starting a fight it may pay to determine as exactly as possible the actual strength of the opponent. In that way it is possible to avoid unnecessary fights if the strength of the opponents differs considerably. We found that strophe length and drift are not only influenced by the male but also by the song type. Only by matching and using a similar song type can opponents exclude the effect of song type on drift. Matching could then be considered to be mechanism of male great tits to estimate each others strength very accurately during the early stages of the territorial period. Territorial fights between birds of unequal strength could then be settled without escalated conflicts (LAMBRECHTS and DHONDT, 1987). This hypothesis is consistent with some of our observations that male great tits can match song types when they are very close together (e.g. within 5 m), i.e. when distance assessment between birds would not be necessary (see above).

Whereas at the behavioural level the predictions relating to the use of singing ability in male-male conflicts were supported, at the population level, and in contrast to former studies, our results suggest that there is no simple relationship between singing ability and territory quality. Thus, males defending a territory in a high quality subplot did not sing longer strophes nor more song types than males with a territory in a low quality subplot. Better singers, therefore, did not have a higher chance to obtain a high quality territory (LAMBRECHTS and DHONDT, 1988b ; LAMBRECHTS *et al.*, submitted). This observation was confirmed when we removed territory owners from their territories in the spring and allowed non-territorial birds to settle in a territory : replacement birds did not produce shorter strophes than birds of the same age that had acquired a territory in a natural way (LAMBRECHTS and DHONDT, 1988b). A possible explanation for this is that good vacant territories are rapidly occupied in late summer, and that locally born first brood males are at an advantage (DHONDT, 1971a). Once a territory is occupied it becomes very difficult to dislodge the owner (KREBS, 1982). Since Great Tits produce two broods that fledge 6 weeks apart, and since local born birds have prior residence to immigrants, possible advantages of male quality may be ofset by disadvantages of later presence. TEUNEN'S (1987) observation that after a sudden massive mortality of the resident males in early January, good singers settled significantly more rapidly than poor singers, supports this idea.

SINGING ABILITY AND REPERTOIRES

Finally, our study of great tit singing performance and the assumption that long strophes are important resulted in a new hypothesis for why birds may need a song repertoire (LAMBRECHTS and DHONDT, 1988a). Great tits that are very motivated will start a song bout with strophes that are much longer than their average strophe length, and these strophes are separated by very short intervals. After a short while the same bird will decrease its strophe length and/or the strophes will be separated by longer intervals. This means that great tits do not only show drift within strophes, but also throughout song bouts. The decrease in song output is more pronounced in shorter bouts, and in bouts that start with longer strophes or with shorter intervals. As for drift within strophes and strophe length, we therefore proposed that drift throughout song bouts is caused by constraints in singing ability. This hypothesis was supported by playback experiments, showing that great tits also show pronounced drift in song bouts in which males were regularly presented with a song stimulus (LAMBRECHTS, 1988 ; WEARY *et al.*, 1991). However, after a switch to a new song type, the birds could increase their song output again to levels that were similar to those observed at the beginning of the previous bout. To explain these findings we proposed that motivated great tits have physiological problems is using the same set of respiratory and syringeal muscles in a stereotyped way for an extended period at high rates. To avoid or reduce neuromuscular exhaustion birds would have to switch to a new song type, in which the respiratory and syringeal muscles are used in another way. Thus, a song repertoire would allow a bird to sing for extended periods at high rates, and high singing rates would increase the success in territorial defence or mate attraction. Our hypothesis does not exclude the idea, however, that great tit song repertoires have more than one function, depending on the context in which birds switch song types. Physiological and morphological studies of the sound apparatus will be necessary to test the « anti-exhaustion » hypothesis in more detail.

CONCLUSION

Initially most of the song studies looked at song characteristics that were mainly influenced by song learning. These studies showed that great tit song provides information on the origin of birds (e.g. song dialects), and on the habitat type in which the birds live. Experimental studies in the field and in laboratory conditions resulted in a series of hypotheses about the functional meaning of song type structure and song repertoires. More recently, even more detailed studies of great tit singing performance revealed that variation in great tit song is not only caused by learning ability and learning opportunities, but could also be influenced by constraints in singing ability. These studies showed that great tit song could reflect male quality, and resulted in a new interpretation of why great tits should have song repertoires. In the future, experiments related to mate attraction of different song lengths, functional studies of great tit singing performance, physiological research of the sound apparatus, and detailed studies of song characteristics that

were not investigated before (*e.g.* volume) are required to know more about the many meanings of great tit song.

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ORGANIZATION OF SONG IN THE EUROPEAN STARLING : SPECIES-SPECIFICITY AND INDIVIDUAL DIFFERENCES

by

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SUMMARY

In this paper we give a detailed description of the song and the song organization of the European starling (*Sturnus vulgaris* LINNAEUS) during the breeding season. We recorded songs of 25 males in Belgium and of 2 males in New Zealand. We show that there are clear species-specific characteristics in the complex song of the starling, but also reveal many individual differences.

During the breeding season, male starlings sing the majority of their song (90 % or more) in long and complex song bouts. These song bouts consist of a rapid succession of a great variety of different song types resulting in a song with much contrast. Although there are large differences in average song bout length among males (range : 15-35 seconds), the song bouts of all males we studied, both in Belgium and New Zealand, were composed of four categories of song types following each other in a fixed order.

Detailed analysis of the sequencing of song types within song bouts of two males showed that song types are not presented randomly : a given song type can be preceded and followed by only a limited number of other song types.

The majority of song types in the repertoires of starlings is individually characteristic. We found large differences in repertoire size among males, extending from 21 to 67 song types.

There appeared to be little difference between Belgian and New Zealand starlings in song organization.

Key-words : European starling, *Sturnus vulgaris*, song, organization of song, song repertoire size, repertoire composition, New Zealand

INTRODUCTION

Nearly all bird species can be identified by their vocalizations (KROODSMA, 1975). Indeed, one of the most consistent and striking characteristics of bird vocalizations is species-specificity. This is related to the fact that most sounds are directed toward conspecifics whose reactions should be appropriate to the content

of the message. Hence, the coding of species-specificity is typically the basis for effective acoustical communication (BECKER, 1982).

Many characteristics of singing contribute to specific distinctiveness among songbirds, but two of the most notable differences among species are the number of different song types per individual (i.e. song repertoire size) and the manner of presentation of those song types during a singing performance (KROODSMA, 1975, 1982).

As clearly stated by KROODSMA (1982), detailed description of the song and the song organization of a species must precede the functional study of song and is a prerequisite to any experimental work. The aim of this paper is to give a detailed description of the song and the song organization of the European starling, *Sturnus vulgaris*. First, we describe in detail the song, the organization of song, the song repertoire size and the composition of the repertoire of 25 male starlings recorded in Belgium. Then, we present data from two males recorded in New Zealand, where European starlings were introduced in 1862 (LONG, 1981 ; BULL *et al.*, 1985).

The complex song of the European starling has only recently received attention (ADRET-HAUSBERGER and JENKINS, 1988 ; EENS *et al.*, 1989). EENS *et al.* (1989) revealed that starling males sing very long and complex song bouts during the breeding season, some of which can extend over one minute in duration. These song bouts consist of a rapid succession of a great variety of different song types. We also showed that starling song bouts have a characteristic sequential organization. However, the previous analysis was based upon a detailed study of only three males (EENS *et al.*, 1989).

STUDY AREAS AND METHODS

Study areas in Belgium

In the first part of this study we provide detailed information on the song organization of 25 male starlings recorded in Belgium. The song of 12 males was recorded in a nest box colony in Zoersel (51°13'N, 4°40'E), near Antwerp, in the breeding season of 1987. Detailed information on the study area, the study population and the general methods can be found elsewhere (PINXTEN *et al.*, 1989a,b, 1990, 1991 ; PINXTEN and EENS, 1990 ; EENS *et al.*, 1991). The song of 13 other males was recorded in large outdoor aviaries located on the campus of the University of Antwerp (51°10'N, 4°25'E) in Wilrijk during the breeding seasons of 1988 and 1989. More details on the outdoor aviaries can be found in EENS *et al.* (1990, 1991). The data from these two groups of males were pooled. This is justified since we have shown that captivity has little or no effect on the singing behaviour of starling males (EENS *et al.*, 1990, 1991). In the Zoersel colony, all males were unmated at the time they were recorded. The captive males were recorded with a female starling in the aviary, as it has been shown that males sing vigorously in the presence of a female (EENS *et al.*, 1990). At the time of recording, males were not yet paired.

All males in the outdoor aviaries and nearly all males in the field were individually marked with wing tags and with colour rings.

Study area in New Zealand

The first European starlings were introduced to New Zealand in 1862 by the 'Nelson Acclimatisation Society'. They are now widespread and abundant in both the North and the South Islands and have reached many of the offshore and outlying islands (LONG, 1981 ; see also BULL *et al.*, 1985).

We recorded the song of several starlings at a semi-isolated colony at Belmont (41°10'S, 174°54'E), near Wellington, New Zealand. The study area is a 1500 ha sheep farm, 250-400 m above sea level, with practically no trees or natural nest sites for starlings within 3 km. The colony was established in 1970 by John and Meg Flux : nest boxes were built into ventilation shafts of abandoned ordnance storage bunkers (see FLUX and FLUX, 1981, 1982 for more details). Between 15 and 17 November 1990 (the breeding season lasts from October to December in this colony), we recorded the song of several males. From one male (male 1) we obtained a large number of high quality recordings enabling us to determine his repertoire size. This male was unpaired at the time of recording. Additional recordings were made from three neighbouring males, one of which was unpaired (male 2), the other two paired at the time of recording. From the latter two males only a few whistles were recorded since they only sang rarely. Each of the four males had a nest box very close to each other in the same bunker (bunker 3). None of the males was individually marked.

Song Recording

Male starlings defend only their nest hole and its immediate surrounding, and this is also where they do most of their singing. All song recordings, both in Belgium and in New Zealand, were made with small electret microphones (Sony ECM-16T or ECM-50 PS) implanted close to or in the nest boxes (see EENS *et al.*, 1989, 1991). These microphones were connected to a Uher 4400 Report Stereo IC tape recorder or a Uher CR 1601 cassette recorder via a long cable. Recordings were made from transportable hides (in the Zoersel colony), from behind one-way glass from permanent observation hides placed close to the outdoor aviaries, or from a car (in New Zealand).

Song Analysis

Song was analysed using a UNIGON 4600 Spectrum analyser (UNISCAN). Hard copy output of all recordings was obtained with an Epson FX-850 printer.

In a previous paper, we showed that male starlings sing very long and complex song bouts during the breeding season (EENS *et al.*, 1989). We define a song bout here as a period of at least five seconds of song containing no pauses longer than 1.5 seconds (modified after HINDMARSH, 1984 ; see EENS *et al.*, 1991). We obtained

on average 26.8 song bouts (SD = 10.7 ; range 12-51) from each of 25 males recorded in Belgium.

Statistical tests follow SIEGEL (1956) and SOKAL and ROHLF (1981). Throughout the paper values given are means ± SD unless stated otherwise. All tests are two-tailed.

RESULTS

General organization of song

Detailed analysis of the song of 25 male starlings recorded in Belgium showed that males sing three categories of song during the breeding season :

1. very long and complex song bouts including heterospecific imitations (see Figs 1 and 2) ;

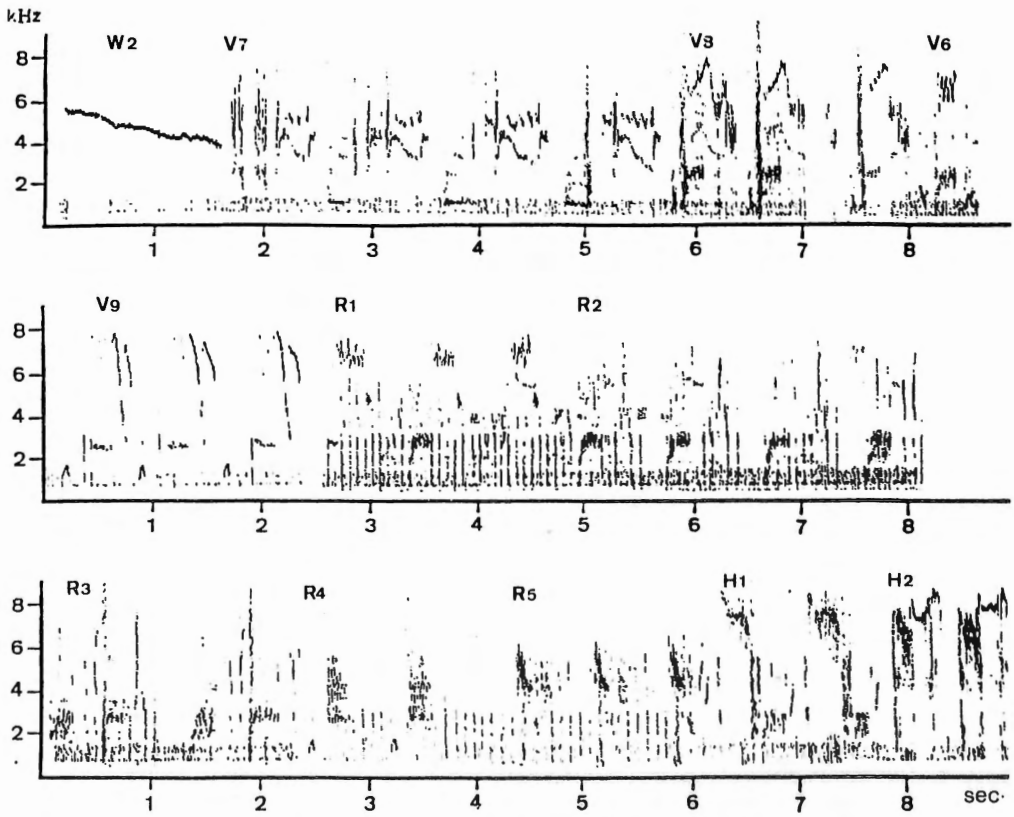

Fig. 1. — Illustration of a complete song bout of male 14F-87 recorded in the colony in Zoersel, Belgium. This song bout lasted 25 seconds and contained 12 different song types: W2 was sung only once, V7 was sung four times, V8 three times etc. (W = whistle, V = variable song type, R = rattle song type, H = high-frequency song type). For more details, see text.

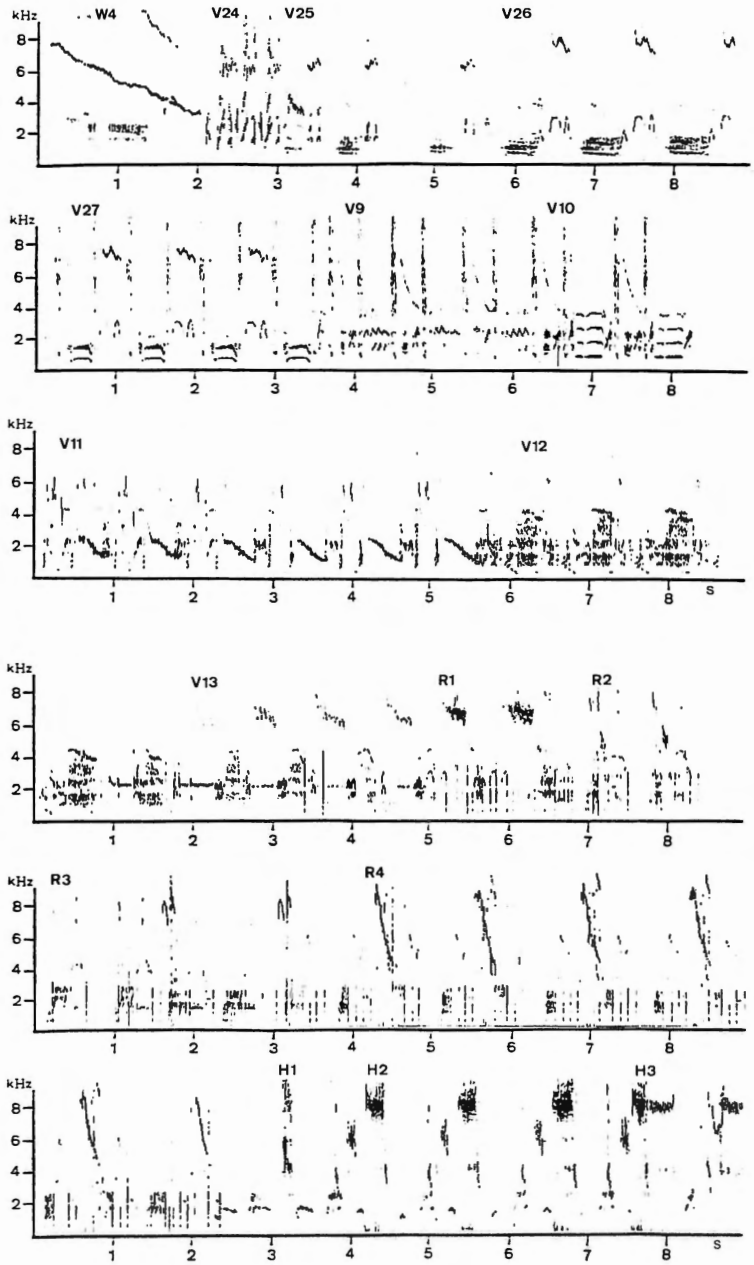

Fig. 2. — Illustration of a complete song bout of male OR-BLA89 recorded in the outdoor aviaries. This song bout lasted 50 seconds and contained 17 different song types (W = whistle, V = variable song type, R = rattle song type, H = high-frequency song type). For more details, see text.

2. simple whistles, occurring between song bouts;
3. imitations of other birds or of non-avian sounds, also occurring between song bouts and never sung as part of a song bout (see Fig. 3A).

Table 1 shows the time devoted to each category of song in the total duration of song analysed for five males (three males of the Zoersel colony and two aviary males were chosen at random). All five males sang the majority of their song (95 % or more) in song bouts. The whistles and the heterospecific imitations that are not sung as part of a song bout account only for a very small percentage (5 % or less) of the total amount of song produced in the breeding season. Note that there is little variation among the five males in this respect (Table 1).

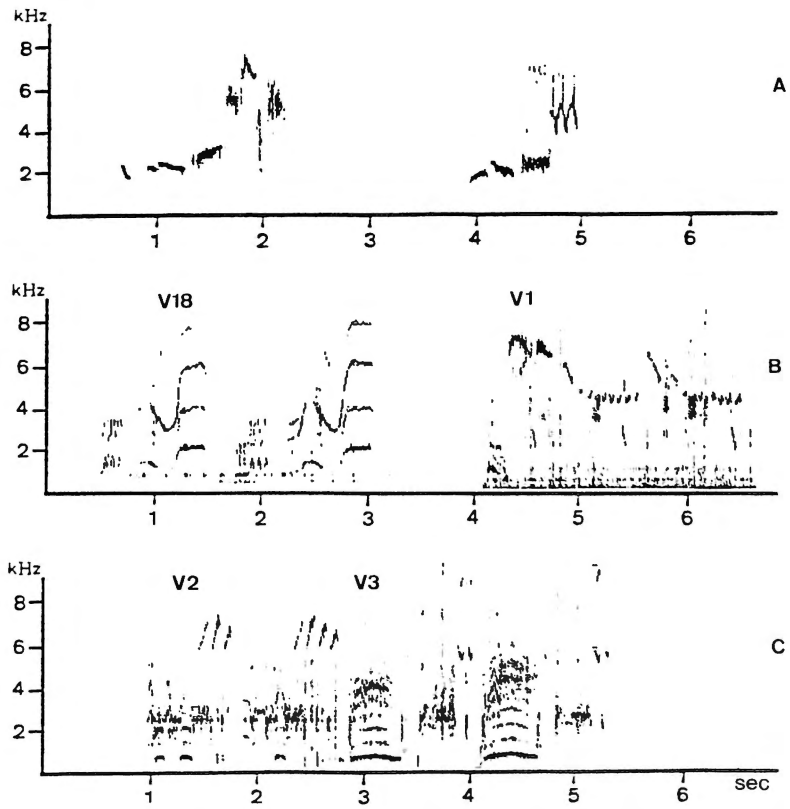

Fig. 3. — Illustration of heterospecific imitations sung by male Starlings. A = two Blackbird imitations by two different males that were sung apart from a song bout. B = two variable song types that are entirely or almost entirely made up of heterospecific imitations: V18 is a lapwing imitation, V1 consists largely of a blue tit imitation. Note also the small differences in successive repetitions of the same imitation. C = two heterospecific imitations that are integrated with other notes in a variable song type: V2 contains a cuckoo imitation, V3 contains a collared turtle dove imitation.

TABLE 1

Time devoted to each category of song in the total duration of song analysed for five males recorded in Belgium. The first three males were recorded in Zoersel in 1987; male four and five were recorded in large outdoor aviaries in Wilrijk.

Male	*Total duration of song analysed*	*Song sung in song bouts*	*Whistles*	*Imitations*
1	1015.3 s	967.4 s (95.3 %)	15.7 s (1.5 %)	32.2 s (3.2 %)
2	867.2 s	839.0 s (96.7 %)	12.7 s (1.5 %)	15.5 s (1.8 %)
3	431.5 s	409.5 s (95.0 %)	11.1 s (2.6 %)	10.5 s (2.4 %)
4	1029.1 s	995.4 s (96.7 %)	13.7 s (1.3 %)	20.0 s (2.0 %)
5	253.7 s	241.1 s (95.0 %)	12.6 s (5.0 %)	0.0 s (0.0 %)

Organization of song bouts

Average song bout length

Average song bout lengths vary considerably among males, ranging from 14.7 to 35.0 seconds. The overall average for 25 males is 24.8 ± 5.4 seconds (see also EENS *et al.*, 1991).

Organization of song bouts

Starling song bouts consist of a succession of song types (or motifs in the terminology of ADRET-HAUSBERGER and JENKINS, 1988), which are fixed combinations of varied acoustic elements (although different repetitions of the same song type can vary slightly : see below and Figs 1 and 2). The majority of song types are repeated immediately once or twice before the next one is sung (see below).

Starling song bouts are composed of four categories of song types, as can be seen in Figs 1 and 2 (see also EENS *et al.*, 1989).

The majority of song bouts begin with one or several 'whistles' (i.e. relatively pure tonelike sounds) : the overall average was 55.1 % (SD = 20.3 ; N = 25 males). The proportion of song bouts that starts with one or several whistles differs highly significantly between males ($G_{williams}$ = 100.7, df = 24, $P < 0.001$) and ranges from 23 % (6 out of 26 song bouts of male OR-BL89 start with a whistle) to 93 % (14 out of 15 in male OR-BL88). Each male has a repertoire of 2 to 11 different whistles, with an average of 6.1 (SD = 2.3 ; N = 25 males). Fig. 4 shows the total repertoire of whistles of male ORX-BL89 : this male sang a total of 11 different whistles. Some whistles can be found in the repertoires of most males (for instance

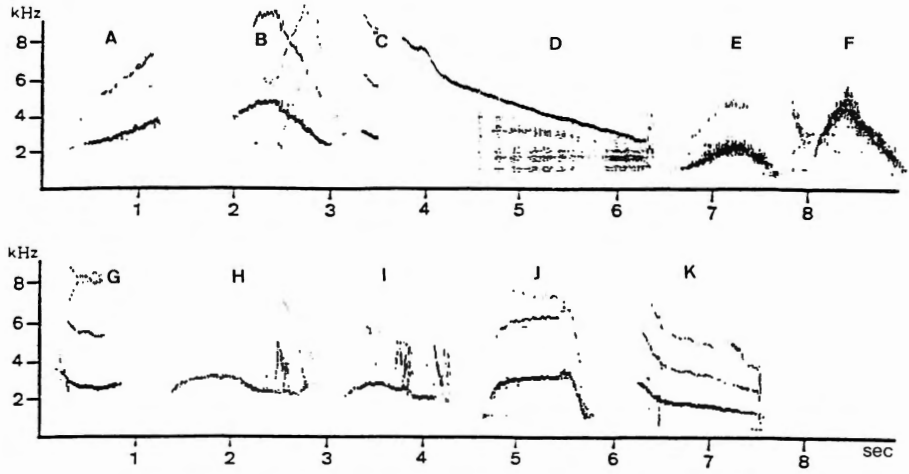

Fig. 4. — Illustration of the total repertoire of whistles of male ORX-BL89. This male sang all together 11 different whistles.

a long descending whistle with an initial frequency of about 8 kHz : compare for instance the introductory whistle in Fig. 2 with the fourth whistle in Fig. 4 (D) and with the whistles shown in Fig. 5), whereas others are unique to the individual. Whistles sung as part of a song bout are mostly not repeated (see Table 2) and are sung rather loudly. As shown in Fig. 5, there can be substantial differences in the exact form of the same introductory whistle. This Figure shows seven variations of

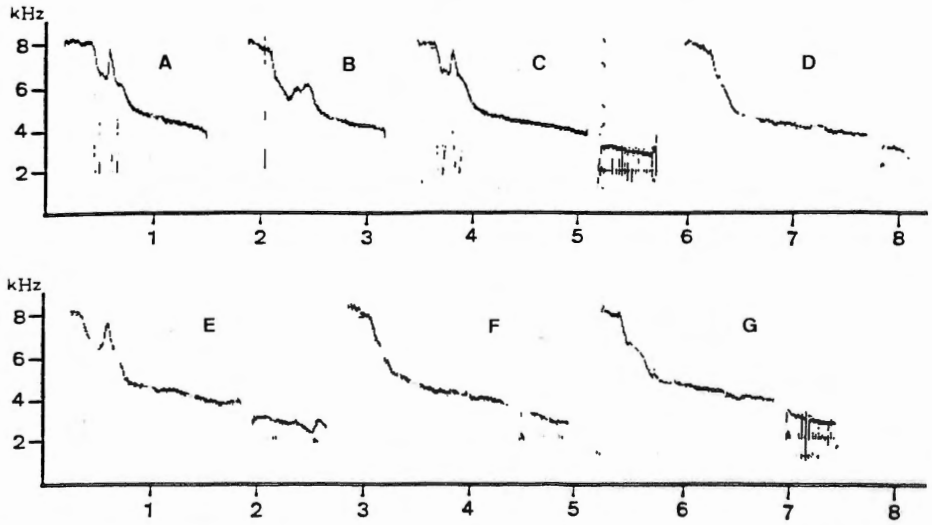

Fig. 5. — Seven song variations on one song type (long descending whistle) of male 1-87 recorded in Zoersel 1987.

the same whistle of a male. It can be seen that there are large differences in the shape of the whistle (compare for instance A with B). Furthermore, in some cases the long whistle is followed by a second smaller one (compare A and B with the rest). There are also large differences in the shape (compare for instance D with E) and the position (sometimes the second whistle is in direct line with the first (F), in other cases not) of the smaller whistle. Finally, in some cases the second whistle is accompanied with a creaking sound produced simultaneously with the whistle (especially C and G : double voicing).

In a song bout, the introductory whistle(s) is (are) always followed by a large number of 'variable song types'. In addition, about 40 % (39.6 % ± 20.1 SD ; range : 7-77 % ; 25 males) of the song bouts start with a variable song type (i.e. the introductory whistle(s) is (are) omitted). Unlike whistles, variable song types have a very complex structure : they usually contain many different notes, many of them covering a wide frequency range in a short time (see Figs 1 and 2). It can be seen that there are large differences in structure between the variable song types of a given individual (see Figs 1 and 2), resulting in a song with much contrast and making it difficult to characterize this category of song type. Variable song types very often contain overlapping notes as a result of double voicing (see for instance most variable song types in Fig. 2 and V2 and V3 in Fig. 3C). Typical for these song types is that they contain most of the heterospecific imitations that are sung in a song bout (see EENS *et al.*, 1989). Some variable song types are entirely made up of heterospecific imitations : V18 in Fig. 3B for instance is entirely made up of an imitation of a lapwing *Vanellus vanellus* (LINNAEUS), whereas V1 (Fig. 3B) consists largely of a blue tit *Parus caeruleus* (LINNAEUS) imitation. In other variable song types, the heterospecific imitations are mixed with other notes : the two notes with a frequency of about 600 Hz in V2 (Fig. 3C) are a cuckoo *Cuculus canorus* (LINNAEUS) imitation (when this song type is sung a second time only one cuckoo note is sung : see Fig. 3C), whereas the note with harmonics in V3 (Fig. 3C) is an imitation of a collared turtle dove *Streptopelia decaocto* (FRIVALDSZKY).

Each male has a repertoire of 10 to 35 different variable song types, with an average of 23.8 (SD = 7.8 ; N = 25 males). Each male has a (nearly) unique reper-

TABLE 2

Average number of times that each of the four categories of song types in a song bout are repeated. To investigate this, we analysed one complete song bout (i.e. in this case a bout containing all four categories of song types; one male who had no high-frequency song types, was excluded from this analysis) of each male (N = 24) *and determined the average number of times that a whistle, a variable, a rattle and a high-frequency song type was sung per male. The table shows the overall average* (X ± SD) *for the 24 males.*

Category	*Whistle*	*Variable song type*	*Rattle song type*	*High-frequency song type*
x ± SD	1.1 ± 0.3	2.3 ± 0.4	2.3 ± 0.4	2.6 ± 0.9

toire of variable song types : compare for instance the variable song types of male 14F-87 (Fig. 1) with those of male OR-BLA89 (Fig. 2). Even neighbouring males nesting only a few meters apart in the same colony, have no variable song types in common. On average, a variable song type is sung 2.3 ± 0.4 times before another is introduced (see Table 2). Variable song types are sung rather quietly.

In a song bout, variable song types pass into what we call 'rattle song types' (see Figs 1 and 2). In addition, four per cent of the song bouts (4.1 % ± 8.5 SD ; range : 0-36 % ; N = 25 males) start with a rattle song type (i.e. without whistles and/or variable song types being sung). Rattle song types are all made up of a rattling sound consisting of a rapid succession of clicks (a click is a short burst of wide frequency noise) with maximum energy below 4 kHz and sung at a rate of about 15 per second ; at the same time several other notes are sung (see Figs 1 and 2). Each male has a repertoire of 2 to 14 different rattle song types, with an average of 8.2 (SD = 3.1 ; N = 25 males). On average, a rattle song type is sung 2.3 ± 0.4 times before another is introduced (see Table 2). Most rattle song types are sung at relatively low amplitude, although rattle song types preceding the final high-frequency song types often are sung louder. Some rattle song types can be found in the repertoires of many males (for instance R5 of male 14F-87 in Fig. 1), whereas others are characteristic of one individual (compare for instance the rattle song types of the two males shown in Figs 1 and 2).

A starling song bout typically ends with a series of high-pitched song types which we call 'high-frequency song types' (see Figs 1, 2 and 6). Frequencies of these song types range mainly from 6 to 10 kHz. High-frequency song types are sung the loudest of a starling song bout. One male out of the 25 did not sing any high-frequency song types despite the fact that we recorded 32 song bouts of this male. Each male sings between 0 and 6 high-frequency song types, with an average of 3.5 (SD = 1.5). One per cent (1.2 % ± 3.2 SD ; range : 0 − 12.5 % ; N = 25 males) of starling song bouts starts with high-frequency song types : in this case the song bouts are mostly made up of high-frequency song types only. On average, a high-frequency song type is sung 2.6 ± 0.9 times before another is introduced (see Table 2). When comparing the high-frequency song types shown in Figs 1, 2 and 6, it can be seen that there are only a limited number of different high-frequency song types or, in other words, most males have one or several high-frequency song types in common. For instance H1 in Fig. 1, H2 in Fig. 6A, H1 in Fig. 6B and H2 in Fig. 6D all are very alike. Four males (three recorded in the field and one recorded in the outdoor aviaries) out of the 25 deviated from the described sequential pattern in that they sometimes returned to rattle song types after the high-frequency song types and eventually again sang high-frequency song types.

Only 30.7 % (SD = 18.4 ; N = 25 males) of the song bouts end with the high-frequency song types due to the fact that the song can be interrupted at any stage during a bout. The percentage of song bouts that ends with high-frequency song types (i.e. complete song bouts) differs highly significantly among males ($G_{williams}$ = 122.2, df = 24, $P < 0.001$), extending from 0 % (0 out of 32 song bouts of male OR-WHI89 ended with high-frequency song types) to 82 % (23 out of 28 in male 8-87).

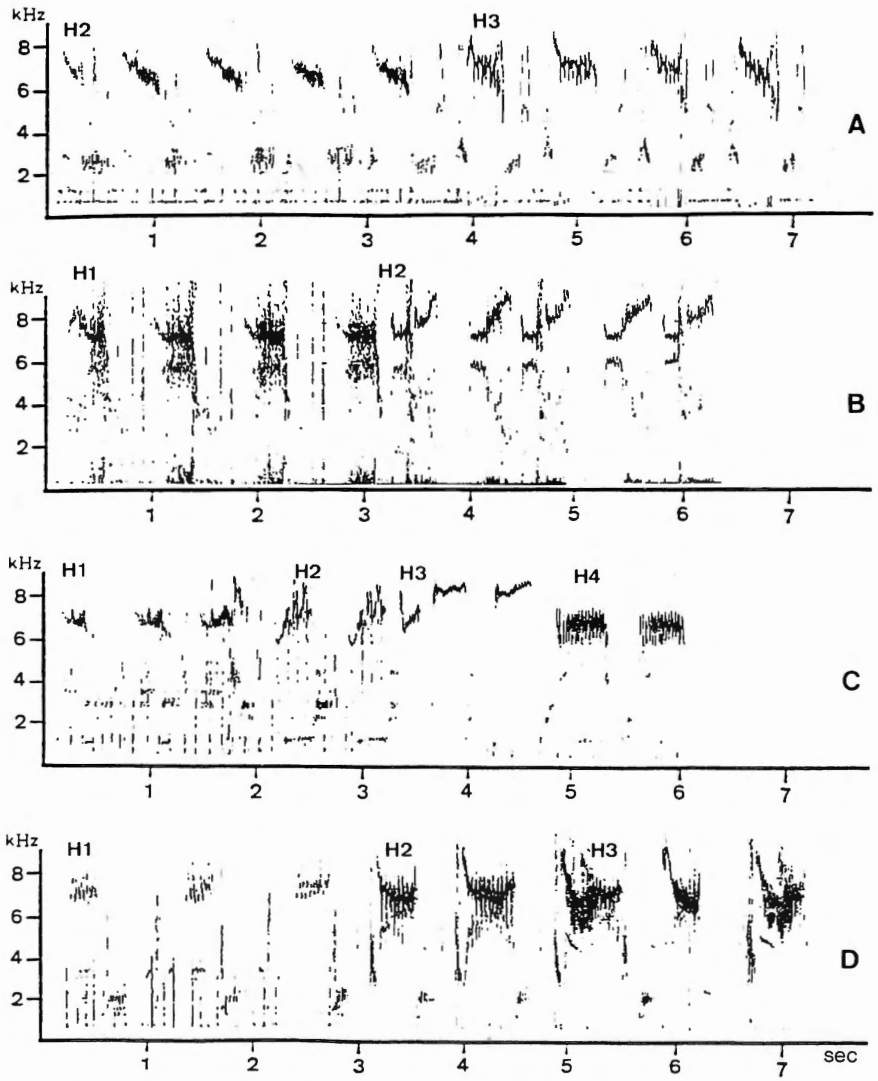

Fig. 6. — Illustration of high-frequency song types found in four different males.

Repetition of song types

To test whether there are differences among males in repetition rate of song types, we analysed one complete song bout of each male (N = 24 since we excluded the male that had no high-frequency song types in this analysis) and determined the average number of times a song type is sung before another is introduced, for each male. We found significant differences among males in the number of times a song type is sung before another is introduced, with averages ranging from 1.8 to 2.8

(Kruskal-Wallis analysis of variance H = 37.2, P = 0.03). The overall average is 2.2 ±0.3 (N = 24).

Detailed sequential organization of song bouts

In Table 3, we provide detailed information on the sequential organization of the song types within the song bouts of two males. These data show that the song types of a given bird are not presented randomly in a song bout. It can be seen that a given song type can be preceded and followed by only a limited number of other song types. If for instance, in the case of male RE-GRE88, a song bout starts with whistles 1 and 2, indicated by W1 and W2, then the variable song types V1, V2, V3, V4 etc. always follow. If, on the contrary, a song bout starts with whistle 3 (W3), then another series of variable song types always follows (see Table 3). So in fact the introductory whistle or whistles (or the introductory variable song type(s)) allow us to predict which succession of song types comes next. It can also be seen that the endings of a song bout are highly stereotyped, irrespective of how the song bouts start. In the case of male RE-GRE88, all complete song bouts end with the rattle song types R5, R6, R7, R8 and high-frequency song types H1, H2, H3. Similarly, the endings of the song bouts of male 11-87 are also highly stereotyped. Stereotyped endings of a song bout are typical for all the starling males we studied.

Although the sequencing of different song types in a song bout is rather stereotyped, a certain amount of variability nevertheless exists. First, a particular song type is sometimes skipped in a sequence of song types : for instance if the typical sequence of song types is V1 V2 V3 V4 V5 V6, then sometimes V1 V2 V4 V5 V6 is sung (the repetitions of a given song type are ignored here). Second, the number of repetitions of a given song type is not always exactly the same from one song sequence to another. Third, when a male switches from one song type to another sometimes intermediate forms occur : for instance if the typical sequence is V1 V1 V1 V2 V2 V2 V3 V3, sometimes V1 V1 (V1V2) V2 V2 V2 V3 V3 occurs whereby V1V2 is composed of a part of song type V1 and a part of song type V2. Fourth, small differences can be found in successive repetitions of a given song type due to the omission or addition of one or a few notes : compare for instance the first and second utterance of variable song type V7 in Fig. 1 and of variable song type V2 in Fig. 3C.

It can be concluded that although starling song bouts have a clear sequential organization, there still is a certain degree of variability. Consequently, a particular male will rarely if ever sing two totally identical song bouts even if the sequence of song types is the same.

Heterospecific imitations not sung as part of a song bout

There are marked differences among males in the number of heterospecific imitations sung apart from a song bout : the number of such imitations ranges from 0 to 11, with an overall average of 1.4 ±2.3. Fig. 3A shows heterospecific imitations of a blackbird *Turdus merula* by two different males. A detailed description

TABLE 3

*Sequential organization of song types within song bouts of two male starlings. Each combination of a letter and a figure stands for a particular song type (*W = *whistle,* V = *variable song type,* R = *rattle song type,* H = *high-frequency song type). The repetitions of a given song type are ignored here. The figure in front of each sequence of song types denotes how many times a given song bout pattern was observed. This presentation is further simplified in two ways. First, as clearly stated in the text, not all song bouts end with the high-frequency song types. Due to lack of space and in order to give a surveyable view of the sequential organization of song bouts, it is not possible to show the organization of each individual song bout of a male nor to indicate at which stage each song bout ended. Second, sometimes a particular song type is skipped in a song bout. (* four song types of male 11-87 (1 whistle, 3 heterospecific imitations) were never sung as part of a song bout).*

Male RE-GRE88 : TOTAL REPERTOIRE SIZE : 31 song types

6 WHISTLES (W)
14 VARIABLE SONG TYPES (V)
8 RATTLE SONG TYPES (R)
3 HIGH-FREQUENCY SONG TYPES (T)

9	W1 (W2)	V1 V2 V3 V4 V5	R1 R2 R5 R6 R7 R8	H1 H2 H3
3		V1 V2 V3 V4 V5	R1 R2 R5 R6 R7 R8	H1 H2 H3
2	W6	V1 V2 V3 V4 V5	R1 R2 R5 R6 R7 R8	H1 H2 H3
6	W5 W4	V6 V7 V8 V9 V10 V11	R3 R4 R5 R6 R7 R8	H1 H2 H3
3	W3	V12 V6 V7 V8 V9 V10 V11	R3 R4 R5 R6 R7 R8	H1 H2 H3
5		V13 V14 V3 V4 V5	R1 R2 R5 R6 R7 R8	H1 H2 H3

Male 11-87 : TOTAL REPERTOIRE SIZE : 46* song types

4 WHISTLES (W)
26 VARIABLE SONG TYPES (V)
8 RATTLE SONG TYPES (R)
5 HIGH-FREQUENCY SONG TYPES (H)

7	(W1) W3	V1 V2 V3 V4 V16 V6 V7 V8	R1 R2 R3 R4 R5 R6 R7	H1 H2 H3 H4 H5
2	W3	V1 V9 V10 V11 V12 V13 V14 V15 V8	R1 R2 R3 R4 R5 R6 R7	H1 H2 H3 H4 H5
1		V24 V25 V26 V10 V11 V12 V13 V14 V15 V8	R1 R2 R3 R4 R5 R6 R7	H1 H2 H3 H4 H5
2	W2	V17 V18 V19 V20 V21 V22 V16 V6 V7 V8	R1 R2 R3 R4 R5 R6 R7	H1 H2 H3 H4 H5
2		V17 V18 V19 V20 V21 V22 V16 V6 V7 V8	R1 R2 R8 R5 R6 R7	H1 H2 H3 H4 H5
7		V23 V5 V6 V7 V8	R1 R2 R8 R5 R6 R7	H1 H2 H3 H4 H5

of the heterospecific imitations sung both in and apart from song bouts awaits further study.

Repertoire size

There were large differences between males in repertoire size, extending from 21 to 67 different song types. The average for 25 males was 42.9 ± 12.4 song types. Fig. 7 shows cumulative plots of new song types against the total number of song types analysed, for four males. It can be clearly seen that the graphs become asymptotic for all four males, doing so more rapidly for the two low repertoire males than for the two high repertoire males. Note that three out of four males still sang new song types after they had already sung 300 song types. One male even sang one new song type after having already sung 800. We analysed 632.5 ± 200 song types (N = 25 males ; range : 325-960 song types) from each male. Consequently, we think we have nearly always obtained the total repertoire size.

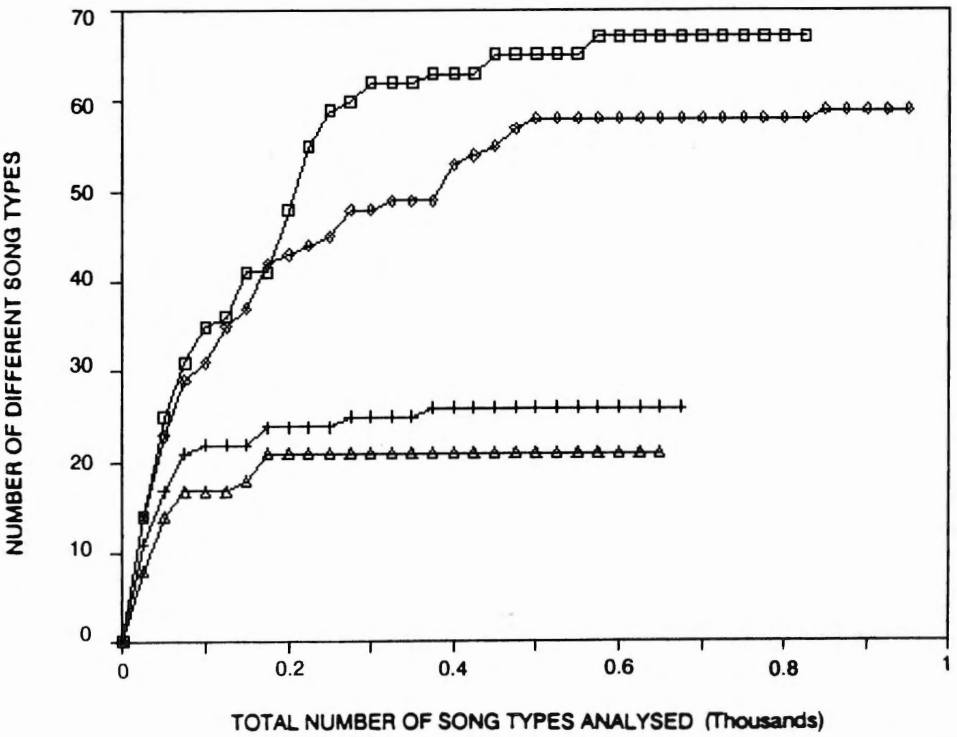

Fig. 7. — Cumulative plots of new song types against the total number of song types analysed for four males. For more details, see text.

We found a highly significant positive correlation between repertoire size and average song bout length (Spearman rank correlation coefficient : r_s + 0.812, N = 25, P < 0.00001 ; see Fig. 8).

There was no difference in the proportion of song bouts that end with high-frequency song types (*i.e.* in the proportion of complete song bouts sung) between low and high repertoire males (low and high refers to the median repertoire size ; Mann-Whitney U-test : $U = 51$, $N_1 = 12$, $N_2 = 12$, $P = 0.23$). There also was no difference in repetition rate of song types between low and high repertoire males (Mann-Whitney U-test : $U = 61.5$, $N_1 = 12$, $N_2 = 12$, $P = 0.54$) nor between males with short and long average song bouts (Mann-Whitney U-test : $U = 53.5$, $N_1 = 12$, $N_2 = 12$, $P = 0.28$).

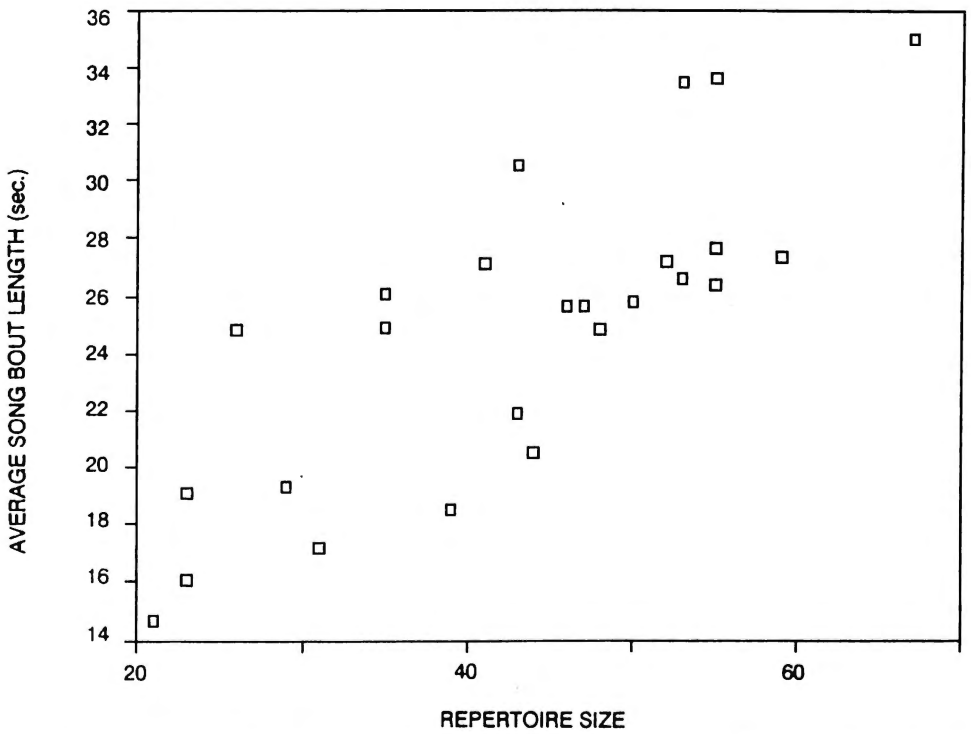

Fig. 8. — Relationship between repertoire size and average song bout length (sec.) for 25 Starling males recorded in Belgium.

Song of New Zealand males

As with the Belgian males, both males recorded in New Zealand sang the majority of their song (> 90 %) in song bouts (Table 4).

Altogether, we recorded ten song bouts of male 1 and eight of male 2. As in the Belgian males, the song bouts of the two New Zealand males were composed of four categories of song types (see Fig. 9). In both males, the majority of song bouts started with a whistle : all song bouts (10 out of 10) of male 1 began with a whistle, while five out of eight (62.5 %) song bouts of male 2 started with a

TABLE 4

Time devoted to each category of song in the total duration of song analysed for two males recorded in New Zealand.

Male	*Total duration of song analysed*	*Song sung in song bouts*	*Whistles*	*Imitations*
1	278.0 s	259.5 s (93.3 %)	18.5 s (6.7 %)	0.0 s (0.0 %)
2	201.8 s	190.7 s (94.5 %)	9.3 s (4.6 %)	1.8 s (0.9 %)

whistle. None of the introductory whistles was repeated in a song bout. In the two New Zealand males we studied, as can be seen in Fig. 9, the introductory whistle was also followed by a long series of variable song types, which in their turn passed into rattle song types. A starling song bout also typically ended with several high-frequency song types. Analysis of one complete song bout of male 1 showed that this male sang a given song type on average 2.2 (SD = 0.6) times before switching to another.

We were able to determine the repertoire size of male 1, from which we analysed in total 342 song types. This male had a repertoire size of 42 different song types and an average song bout length of 25.95 seconds (SD = 12.7; N = 10 song bouts). Male 1 had 10 whistles, 18 variable song types, 9 rattle song types and 5 high-frequency song types in his repertoire. Two variable song types of male 1 contained an imitation of an Australian magpie (*Gymnorhina tibicen* CAMPBELL). Male 2 had an average song bout length of 23.9 seconds (SD = 14.2, N = 8 song bouts). We were unable to determine his repertoire size due to the low quality of the recordings as a result of the windy conditions at the time of recording. At least two variable song types of male 2 contained a heterospecific imitation : one contained a call of a common chaffinch (*Fringilla coelebs* LINNAEUS), the other a call of a house sparrow (*Passer domesticus* LINNAEUS). Male 2 also sang a blackbird imitation though not in a song bout. All four species mentioned have been introduced into New Zealand.

As starlings have large repertoire sizes and as there are large differences in the exact structure of song types between male starlings even from the same colony and nesting only few meters apart (see above), it is difficult to compare song structures from males of different populations in a quantitative manner. However, using a more qualitative approach, some interesting points can be raised. First, both male 1 and 2 of New Zealand often started a song bout with a long descending whistle with a beginning frequency of about 8 kHz (see Fig. 9) : four out of ten song bouts of male 1, and two out of five song bouts of male 2 started with such a whistle. This type of whistle is also the most frequently used introductory whistle in Belgian starlings (compare whistle W6 of male 1 in Fig. 9 with Figs 2, 4D and 5). Second, eight of the nine other whistles of male 1 were of a type also found in Belgian star-

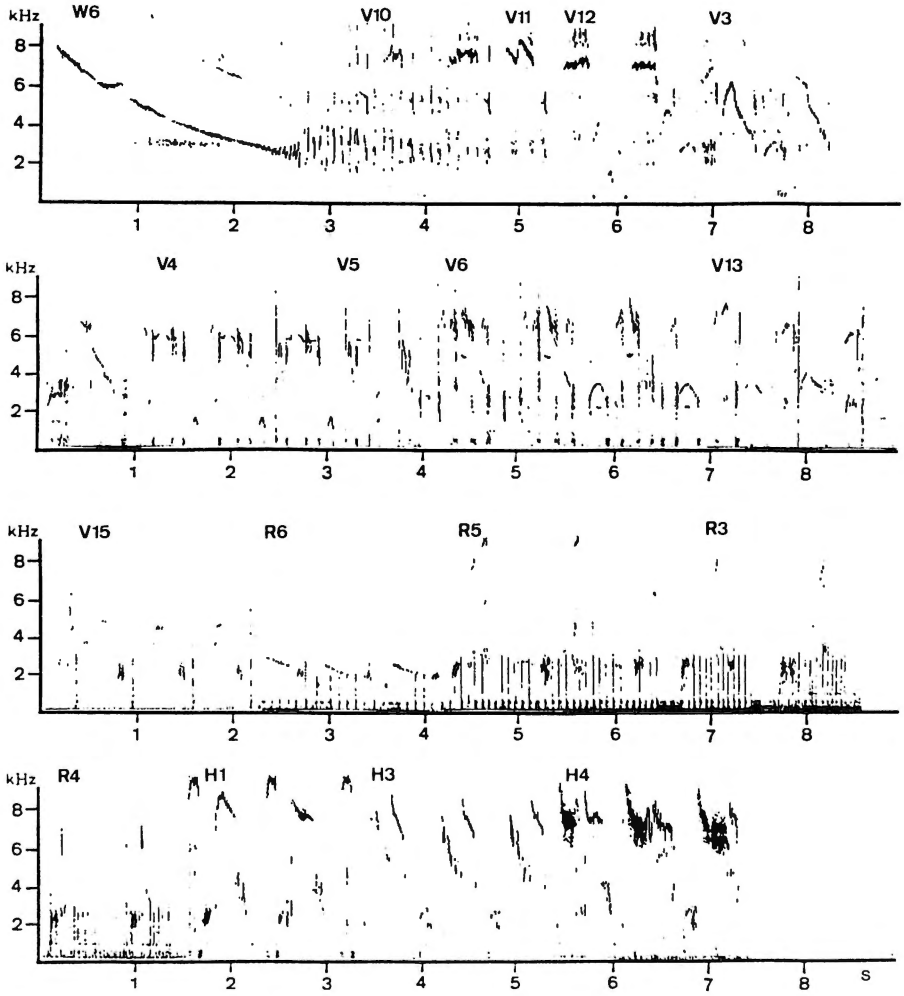

Fig. 9. — Illustration of a complete song bout of male 1 recorded in New Zealand. This song bout lasted 34 seconds and contained 17 different song types. (W = whistle, V = variable song type, R = rattle song type, H = high-frequency song type). For more details, see text.

lings. Only one whistle of male 1 was of a type never found in Belgium : interestingly, all four males of bunker 3 sang this whistle (see Fig. 10). Third, the variable and rattle song types of male 1 were very similar to those found in Belgian starlings except, of course, for the heterospecific imitations of the Australian magpie (compare Figs 1 and 2 with Fig. 9). Fourth, most of the high-frequency song types recorded in New Zealand were very similar to those found in Belgium : for instance H1 in Fig. 9 is similar to the first H2 in Fig. 6A, while H4 in Fig. 9 is very similar to H3 in Fig. 6D.

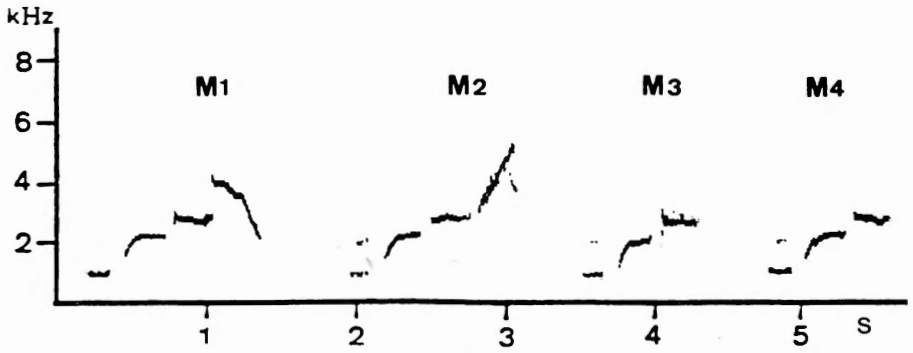

Fig. 10. — Illustration of a whistle type not found in Belgium, that was sung by all four males of bunker 3 in New Zealand. Male 1 and 2 (M1 and M2) sometimes omitted the fourth note when singing this whistle.

DISCUSSION

The European starling has an extremely complex and varied song. We showed that the majority of song, both in Belgium and New Zealand, is sung in song bouts during the breeding season. These song bouts, which in some males can extend over one minute in duration and which have an average duration of about 25 seconds (N = 25 males recorded in Belgium), consist of a rapid succession of a great variety of different song types resulting in a song with much contrast. Our study showed some clear species-specific characteristics in the starling song, but also revealed many individual differences (see also EENS *et al.*, 1989).

Although there are large differences in average song bout length and in song repertoire size between males, the song bouts of all males we studied, both in Belgium and New Zealand, are composed of four categories of song type. The starling song is an uninterrupted sequence of structurally complex song types, progressing from relatively simple pure-tone whistles to the more complex variable and rattle song types and ending with high-pitched trills. We found that, both in Belgium and New Zealand, the introductory whistles are mostly not repeated. Song types of the other three categories are mostly repeated. The number of repetitions of song types differed between Belgian males, with averages ranging from 1.8 to 2.8. The one New Zealand male we analysed sang a given song type on average 2.2 times before switching to another, suggesting that the tendency to repeat a given song type before singing another is typical for all starlings (see also ADRET-HAUSBERGER and JENKINS, 1988).

When comparing the song of Belgian and New Zealand starlings, there appeared to be little difference in the organization of song, the general characteristics of the four categories of song type or in the structure of many song types (compare Figs 1 and 2 with Fig. 9). We found for instance that both in the Belgian and New Zealand males we studied, a long descending whistle was often used as an introductory whistle. It is also interesting to note that some variable song types

of the New Zealand males contained heterospecific imitations, which is in agreement with our results from Belgium (EENS *et al.*, 1989 ; this study). One whistle (exclusively sung apart from song bouts) which was found in all four males recorded (Fig. 10) was of a type never found in Belgium. However, since we are not familiar with the song of all New Zealand birds, it is not unlikely that it was a heterospecific imitation.

Detailed analysis of the sequencing of song types within the song bouts of two males showed that starling song bouts are determinate to a great extent (cf. EENS *et al.*, 1989), although we also showed that a certain degree of variability always occurs.

Although four categories of song type can be found in the song bouts of all males, most song types in the repertoires of starling males are individually characteristic. This raises the question how song repertoires develop in the starling. All songbirds studied to date have been found to learn details of their songs from other individuals (SLATER, 1989). The observation that a large part of the repertoire of each starling from the same colony, even if nesting only a few meters apart, is unique suggests that starlings probably do not learn songs from their neighbours or learn them very inaccurately. Since starlings spend most time of the year in flocks (FEARE, 1984), especially in a non-resident population, such as the one of Zoersel, it is not unlikely that juvenile starlings learn their song from many different models. Another, not mutually exclusive, possibility might be that the individuality of song is to some extent the result of improvisation (MARLER and PETERS, 1982 ; see EENS *et al.*, 1989). As yet, little is known about the timing of vocal learning in the starling. BÖHNER *et al.* (1990) recently found that starlings are able to learn new songs at an age of 11 to 12 months.

Our results on the organization of song in the starling differ in several respects with those found by ADRET-HAUSBERGER and JENKINS (1988) who recorded starling songs in France and New Zealand. First, although they gave no exact figures, ADRET-HAUSBERGER and JENKINS (1988) found that starling song bouts always start with an 'individual motif', and never with a whistle (or several whistles). Second, although they also found 'high pitched trills' at the end of song bouts, their results suggest that each male has only one or two of these song types. We found an average of 3.5 high-frequency song types per male with individual values ranging from 0 to 6. Male 1 recorded in New Zealand sang a total of 5 high-frequency song types. Third, whereas we found an overall repertoire size of about 43 song types with individual repertoire sizes ranging from 21 to 67 song types, ADRET-HAUSBERGER and JENKINS (1988) found repertoires ranging from 11 to 36 song types with an overall average of 23.6 song types (SD = 7.5 ; N = 14 males). ADRET-HAUSBERGER and JENKINS' more limited recording time per male may have resulted in an underestimation of repertoire size and this may account for the latter two differences (see EENS *et al.*, 1991). The first difference, however, might suggest that there may be differences among populations in the organization of song. Another possibility might be that the differences between both studies are due to differences in the timing of recording. We recorded the songs of males at the time they were still unmated. Since ADRET-HAUSBERGER and JENKINS (1988) did not individually

mark their males, they probably had no information on the mating status of their males at the time of recording. In the mockingbird (*Mimus polyglottos*) it has been found that the social situation in which the singing behaviour occurred can alter significantly the estimate of repertoire size : DERRICKSON (1987, 1988) found that estimated repertoire sizes are larger for male mockingbirds when associated with females than in agonistic situations. Since the song of the starling seems to function largely in male-female interactions (CUTHILL and HINDMARSH, 1985 ; EENS *et al.*, 1990 ; EENS and PINXTEN, 1990), it is not unreasonable to assume that the recording of the song of already-mated males might lead to an underestimation of the song repertoire size.

Worth mentioning is the observation that starlings can sing over a large frequency range. The cuckoo and collared turtle dove imitations shown in Fig. 3C for instance have a minimum frequency of about 6OO Hz while some of the high-frequency song types can have a frequency of 10 kHz or even more. We know of no other European songbird species that is able to sing frequencies as low as the starling. The marsh warbler *Acrocephalus palustris* for instance which has a capacity for heterospecific mimicry unequalled by any other species studied so far (LEMAIRE, 1974 ; Dowsett-Lemaire, 1979), is unable to imitate the sounds emitted by the *Colombidae* and the cuckoos, since their low-pitched voices fall outside its frequency range (1500-8000 Hz) (LEMAIRE, 1974 ; DOWSETT-LEMAIRE, 1979). As demonstrated above, this is not the case in the starling.

We showed that there is considerable inter-individual variation in average song bout length and in repertoire size between males. Average song bouts ranged from about 15 to 35 seconds, while repertoire sizes ranged from about 20 to almost 70 song types. Since we recorded a large amount of song from all males, we have probably nearly always obtained the total repertoire size. However, it should be noted that in the repertoire of a given male, some song types are relatively common, while others are sung very rarely : in the case of male 11-87 (see Table 3) for instance, variable song type V8 occurred in every song bout, whereas variable song types V24, V25 and V26 were found only once. Therefore, the possibility that some rare song types were not found by us cannot completely be excluded.

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FIBRE COMPOSITION OF THE MASTICATORY MUSCLES OF *PTEROPUS GIGANTEUS* (BRUNNICH, 1782) (MEGACHIROPTERA)

by

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SUMMARY

Routine histochemistry was used to study fibre type composition of the masticatory muscles of the frugivorous flying fox, *Pteropus giganteus* (BRUNNICH, 1782). Frozen sections were stained for alkaline- and acid-stable ATPase, NADH-tetrazolium reductase and α-glycerophosphate dehydrogenase, and fibres were subsequently identified as slow-twitch oxidative (SO), fast-twitch oxidative glycolytic (FOG) and fast-twitch glycolytic (FG). Based upon relative proportions of fibre types, muscles and their subdivisions can be classified into three groups : group 1 muscles (superficial and medial temporales), containing less than 10 % of SO fibres, group 2 muscles (superficial and deep masseter, zygomaticomandibularis, deep temporalis, medial pterygoid), containing 20-30 % of SO fibres, and group 3 muscles (anterior and posterior digastrics), containing 30-50 % of SO fibres. Moreover, in group 3 muscles less than 5 % of the fast twitch fibres are fatigue resistant (FOG), whereas in both group 1 and 2 muscles, about 20-30 % of the fast-twitch fibres are FOG.

The histochemical profile of the masticatory muscles is correlated directly with their contraction characteristics and indirectly with their EMG patterns.

Key words : histochemistry, contraction characteristics, masticatory muscles

INTRODUCTION

The wide range of mechanical demands imposed upon the masticatory apparatus of mammals is reflected in its structural and functional diversity. Despite detailed descriptions of the anatomy of skull and mandible, and analyses of patterns of jaw movements and coincident muscle activity in a wide variety of mammals (DE GUELDRE and DE VREE, 1988), relatively few studies consider the histochemical profile of the jaw muscles involved in chewing in their explanation of function. However, studies on limb muscles have indicated that fibre type distribution is related to contractile properties (*e.g.* BURKE, 1978 ; CLOSE, 1972), and hence may

have consequences on attempts to explain the mechanics of movements and their neural control.

The morphology and mechanics of the masticatory apparatus of the flying fox, *Pteropus giganteus* (DE GUELDRE and DE VREE, 1984, 1988, 1990) are adapted to a diet of soft fruit pulp and fruit juices . The aim of the present study was to investigate how this is translated in the fibre type composition of the main masticatory muscles. The histochemical fibre types were obtained by routine staining techniques. Isometric contraction properties of some muscles were also investigated by direct stimulation of whole muscles in vivo.

MATERIAL AND METHODS

Histochemistry

Muscle biopsies were obtained from four freshly killed adult *Pteropus giganteus* (BRUNNICH, 1782) (460-500 g), obtained from a commercial dealer from India. Bundles were excised from the middle of the superficial and deep masseter, zygomaticomandibularis, superficial anterior, superficial posterior, medial and deep temporales, medial and lateral pterygoid, and anterior and posterior digastric muscles. Topography and anatomy of these muscles were described in detail previously (DE GUELDRE and DE VREE, 1988). The bundles were rapidly frozen in isopentane, precooled with liquid nitrogen, and subsequently stored at -70° C until required (within 14 days).

From each muscle series of transverse sections (8 μm) were cut in a cryostat (-22° C), mounted on dry slides and air-dried. Subsequent sections of each series were stained for one of the following enzymes (methods see DUBOWITZ, 1985) : alkaline-stable myofibrillar adenosine triphosphatase (mATPase) by preincubation for 15 minutes at pH 9.4 and incubation for 30 min (37° C), acid-stable mATPase by preincubation for 5 minutes at pH 4.6 or 4.35 and incubation for 45 minutes, reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) with an incubation time of 30 minutes, and menadione linked α-glycerophosphate dehydrogenase (α-GPD) with an incubation time of 60 minutes.

From each series of sections, a representative field of at least 200 fibres was chosen, and the numbers and positions of positively reacting fibers determined for each of the different enzymes. Muscle fibres were classified as slow-twitch oxidative (SO), fast-twitch oxidative glycolytic (FOG), and fast-twitch glycolytic (FG) (PETER *et al.*, 1972). The values of table 1 represent the means (and standard deviation) of the four animals. However, for the posterior part of the superficial temporalis, deep temporalis and lateral pterygoid muscles only one sample was available.

Contraction characteristics

In vivo stimulation of the medial temporalis, superficial masseter and zygomaticomandibularis muscles was performed on three specimens of *Pteropus giganteus*. The animals were anesthetized with Ketalar (100 mg/kg, IM) and Rom-

pun (0.4 mg/kg, IM). Deep anesthesia was maintained by further administration of Ketalar during the experiment as necessary. The muscles to be studied were exposed bilaterally and unipolar stainless steel electrodes were inserted near their origins and insertions with the aid of 16-gauge hypodermic needles. The free ends of the electrodes were connected to a stimulator (Grass S48). Subsequently, the cranium of each animal was fixed with the occlusal plane of the upper teeth horizontally, and an isometric force-displacement transducer (Grass FT10) was attached under and to the ventral side of the mandible in a horizontal position.

Supramaximal single square wave stimuli (2 ms duration, 5-8 V intensity) or stimulus trains (1-50 Hz) were applied to each muscle bilaterally. The mechanical response (twitch or tetanus) was recorded by means of the isometric force transducer as closing force exerted by the mandible . The signal was passed through a Gould D.C bridge amplifier (Model 13 4312 00), and finally displayed on a Tektronix R 5103 N storage oscilloscope and a Gould (Brush 481) multichannel chart recorder. The output was calibrated by 100-500 g loads. A series of muscle twitches, elicited by stimuli applied at a rate of 1/s, were recorded for each of different jaw positions, ranging from near closure, over wide open, to near closure again. Jaw positions were measured with dividers as the distance between the incisors. Tetanisation was studied in each muscle at the jaw position which gave a maximum twitch.

Each experiment yielded a set of data for each muscle. Twitch duration and time-to-peak-tension of single twitches, as well as peak amplitude at different jaw positions, were determined. Furthermore, tetanic frequency, rate of tension rise and peak tetanic tension were also obtained. Since the transducer measured the closing force of the tip of the mandible (which in fact are measurements of the outforce of the lever system and, hence, have no absolute meaning) tension values were expressed as the percentage of maximum tension, obtained at optimal jaw position (ANAPOL and HERRING, 1989). To allow comparisons among experiments, length measurements were expressed as percentages of optimal jaw position. Finally, initial muscle length as well as the change in muscle length during jaw opening were calculated indirectly for each of the jaw muscles studied from the origin-insertion distance with the jaws closed and the change of this distance at each of different jaw positions.

RESULTS

Histochemistry

At least 200 fibres are studied for each muscle. Three distinct fibre types are identified in all muscles on the basis of histochemical staining intensities. One type consists of large fibres, that stain darkly for alkaline-stable mATPase and α-GPD, and lightly for NADH-TR. These histochemical characteristics are consistent with the fast-twitch glycolytic (FG) fibres (PETER *et al.*, 1972). Smaller fibres with a strong NADH-TR and acid-stable mATPase activity, and a weak α-GPD activity correspond with the slow-twitch oxidative (SO) fibres. The remaining intermediate

sized fibres show moderate to strong NADH-TR and α-GPD, and strong alkaline-stable mATPase activity. They conform to the fast-twitch oxydative glycolytic (FOG) fibres. However, as contrasted to limb muscle, the FOG fibres were not readily identifiable after acid preincubation at pH 4.6.

All masticatory muscles of *Pteropus* contain a higher proportion of fast-twitch fibres as compared to the proportion of slow-twitch fibres (Table 1). However, the proportions of each fibre type differ among muscles and muscle subdivisions. Three groups of muscles can be distinguished. The muscles of the first group (group 1) mainly consist of fast-twitch fibres (more than 90 %), more than 60 % of which are of the FG type. Group 1 muscles contain the lowest proportion of slow-twitch (SO) fibres (less than 10 %). To this group belong the superficial and medial temporales. The second group of muscles (group 2) is characterized by 20-30 % of slow-twitch (SO) fibres and 70-80 % of fast-twitch fibres. Of the latter, 20-25 % belong to the FOG type and 45-55 % to the FG type. To this group belong the superficial anterior and deep masseter, zygomaticomandibularis, deep temporalis and medial pterygoid. The muscles of the third group (group 3) contain the highest proportion of slow-twitch (SO) fibres (more than 30 %) and the lowest proportion of fast-twitch fibres, less than 5 % of which belong to the FOG type. The anterior and posterior digastric belong to this group. Two muscles cannot be classified in either of the three groups. The posterior part of the superficial masseter contains a lower proportion of slow-twitch (SO) fibres, and hence, a larger proportion of fast-twitch fibres, as compared to the anterior part of this muscle. On the other hand, the lateral pterygoid exhibits a special fibre composition. This muscle shows similar characteristics as the group three muscles (digastrics) in the division between slow- and fast-twitch fibres. However, all the fast-twitch fibres of this muscle appear to belong to the FOG type.

Contraction characteristics

Contraction characteristics are obtained from muscles representative for the histochemically distinct group 1 (medial temporalis) and group 2 (superficial masseter and zygomaticomandibularis). In all muscles studied, the twitches are fast. However, the twitch characteristics differ among muscles. Time-to-peak-tension is significantly different ($P < 0.05$) in all muscles, being longest in the masseter (36.3 ± 3.8 ms) and shortest in the temporalis (21.7 ± 2.4 ms); the zygomatico-mandibularis (28.8 ± 1.25 ms) shows intermediate values. The twitch duration changes similarly (masseter, 68.8 ± 1.25 ms; zygomaticomandibularis, 56.3 ± 3.8 ms; temporalis, 55.0 ± 5.0 ms respectively), but is only significantly longer in the masseter as compared to the other muscles.

The length-tension relationship of single twitches in terms of gape indicates that the different muscles produce their maximum tension with the jaw opened; the associated average muscle lengths at optimum jaw position amount respectively to 1.26 times the initial muscle length in the medial temporalis muscle, 1.22 in the masseter muscle, and 1.29 in the zygomaticomandibularis muscle (Fig. 1). The optimum jaw position coincides with the maximum jaw position observed during

TABEL 1

Mean percentage distribution (± SD) *of slow-twitch oxidative* (SO), *fast-twitch oxidative glycolytic* (FOG) *and fast-twitch glycolytic* (FG) *fibres in the main masticatory muscles and their subdivisions of* Pteropus giganteus.

Muscle	SO	FOG	FG	FOG + FG	FG/FOG	FG/FOG + SO
Superficial masseter						
anterior	28.3 ± 3.3	22.1 ± 2.1	49.5 ± 4.8	71.5 ± 3.3	2.3 ± 0.4	1.0 ± 0.2
posterior	15.5 ± 2.9	26.3 ± 0.0	58.3 ± 3.0	84.6 ± 3.0	2.2 ± 0.1	1.4 ± 0.2
Deep masseter	27.4 ± 4.3	18.1 ± 2.6	54.6 ± 2.3	72.6 ± 4.3	3.1 ± 0.4	1.2 ± 0.1
Zygomaticomandibularis	22.1 ± 2.4	22.3 ± 3.0	55.6 ± 7.2	77.9 ± 2.4	2.8 ± 1.3	1.3 ± 0.4
Superficial temporalis						
anterior	6.6 ± 0.7	28.0 ± 0.3	65.4 ± 0.9	93.4 ± 0.6	2.3 ± 0.1	1.9 ± 0.1
posterior	1.3	37.2	61.4	98.6	1.6	1.6
Medial temporalis	8.4 ± 0.0	26.8 ± 2.7	64.8 ± 2.7	91.6 ± 0.0	2.5 ± 0.3	1.9 ± 0.2
Deep temporalis	25.4	26.8	47.9	74.7	1.8	0.9
Medial pterygoid	27.9 ± 2.3	22.5 ± 5.3	49.6 ± 4.1	72.1 ± 2.3	2.4 ± 0.9	1.0 ± 0.2
Lateral pterygoid	48.8	51.2	0.0	51.2	0.0	0.0
Anterior digastric	31.1 ± 2.7	4.2 ± 3.0	64.7 ± 1.2	68.9 ± 2.7	23.4 ± 9.3	1.8 ± 0.1
Posterior digastric	47.3 ± 1.1	3.4 ± 0.6	49.4 ± 0.8	52.7 ± 1.1	15.2 ± 2.5	1.0 ± 0.0

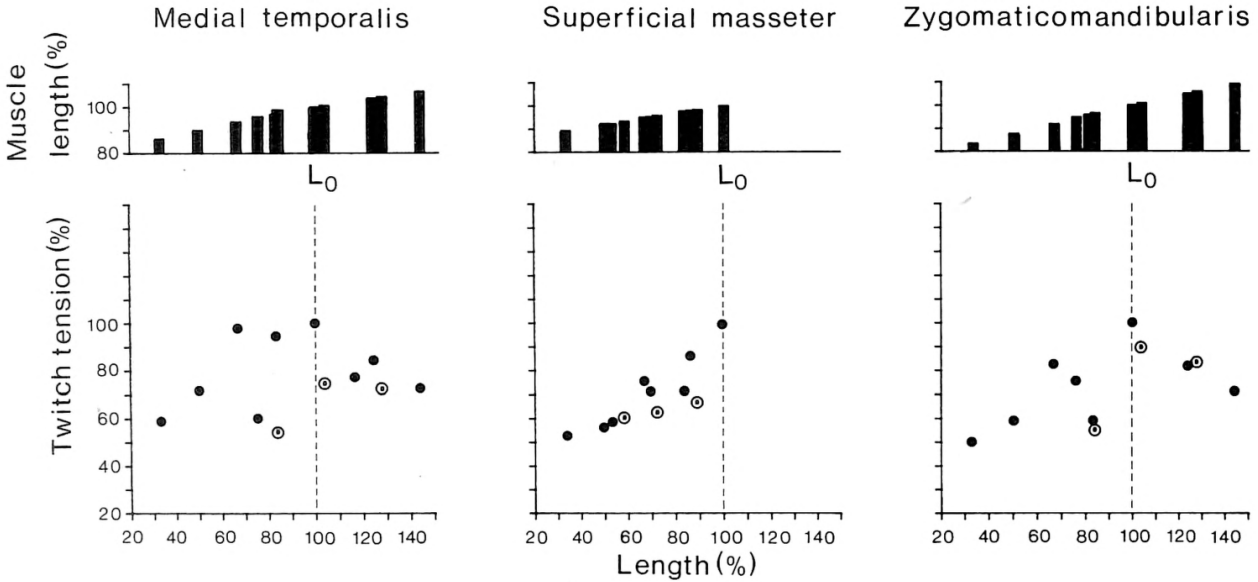

Fig. 1. — Length-tension relation of isometric twitches elicited by stimuli at a rate of 1 Hz at increasing (●) or decreasing (⊙) length (lower trace) and associated mean muscle lengths (upper trace) in medial temporalis, superficial masseter and zygomaticomandibularis muscles. Length is measured as the distance between the incisors and expressed as the percentage of optimum distance. Tension is expressed as the percentage of maximum tension obtained at optimum jaw position. Mean muscle lengths at each of different jaw positions are expressed as percentages of optimum muscle length L_0. L_i, initial muscle length (with closed jaws).

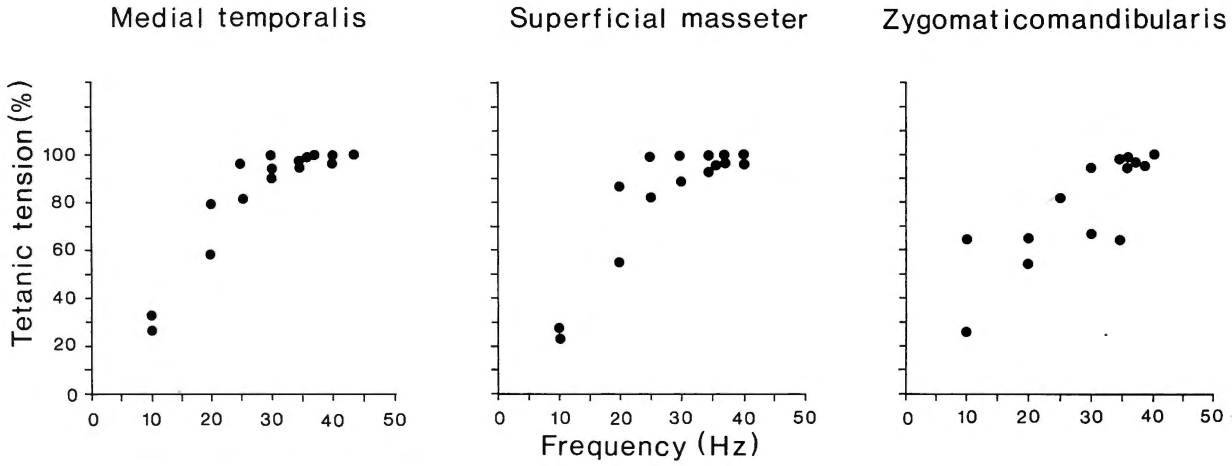

Fig. 2. — Frequency-tension relation of tetanic contractions elicited by stimulus trains of increasing rate at the optimum jaw position in medial temporalis, superficial masseter and zygomaticomandibularis muscles. Tension is expressed as the percentage of maximum tetanic tension.

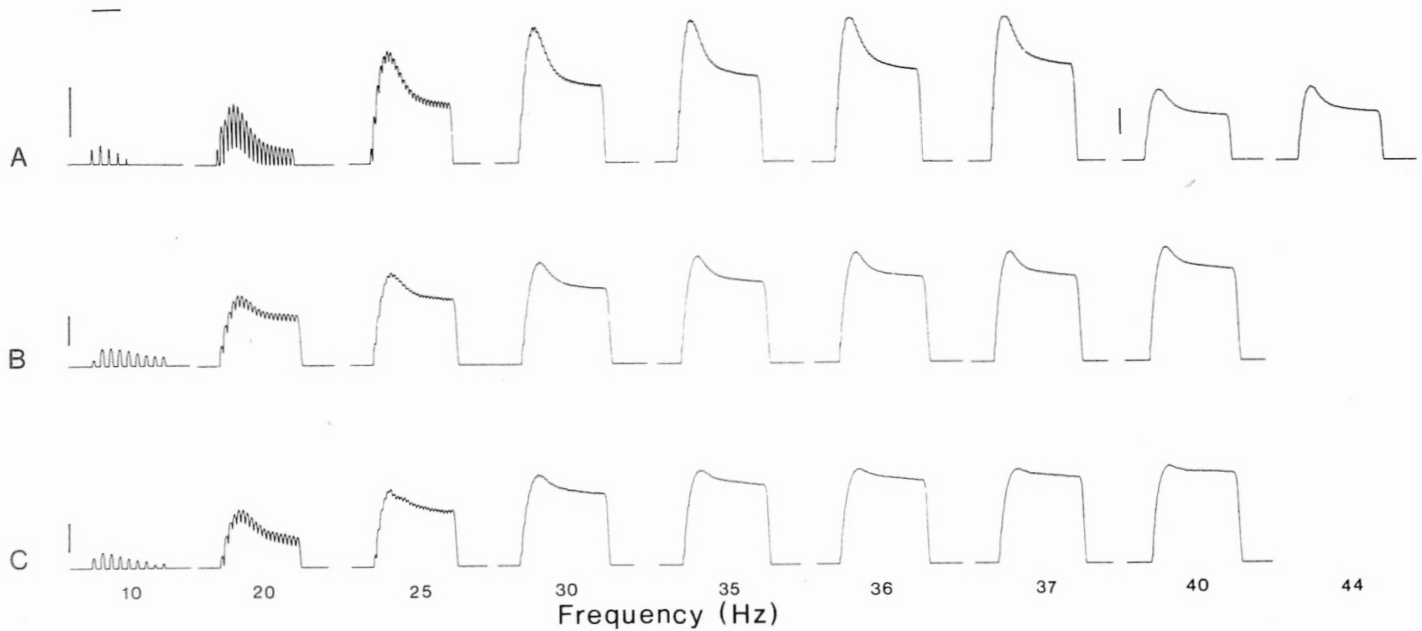

Fig. 3. — Representative isometric responses to stimulus trains of increasing frequency, elicited at optimum muscle length in medial temporalis (A), superficial masseter (B), and zygomaticomandibularis (C). Horizontal line, 200 ms ; vertical line, 100 g.

normal function in the masseter muscle, but is slightly below this maximum in the other two muscles. It is not clear whether this is an artefact of the method used. Furthermore, a slight hysteresis effect is observed in all muscles examined; the measured tension is generally less during stepwise shortening of the muscle after previous lengthening of the muscle, than during lengthening itself.

Maximum tetanic tension, elicited at the optimum muscle length, is reached at a frequency between 37-43 Hz (Fig. 2). This frequency coincides with the threshold frequency for smooth tetanic tension in all three muscles. The rate of tetanic tension rise increases with increasing frequency in the temporalis (time-to-peak tetanic tension decreases from ±240 ms to ± 160 ms in the range from 20-45 Hz); in the masseter and zygomaticomandibularis muscles, the time-to-peak tetanic tension remains ± 220 ms (Fig. 3).

DISCUSSION

Fibre types

Most information obtained to date on the histochemical fibre composition of masticatory muscles in mammals indicates their heterogeneous nature, and reveals considerable variation in proportions and cross-sectional areas of each fibre type, both within and among species (BOSLEY *et al.*, 1972; CLARK and LUSCHEI, 1981; GORNIAK, 1986; HERRING *et al.*, 1979; HIIEMAE, 1971; HIRAIWA, 1978; KITA, 1971; MASUDA *et al.*, 1974; MAXWELL *et al.*, 1979; RINGQVIST, 1974; RINGQVIST *et al.*, 1977; SCHIAFFINO, 1974; SUZUKI, 1971, 1977; TAYLOR, 1976; TAYLOR *et al.*, 1973). Intraspecific variation within muscles appears to be correlated with sexual dimorphism in craniofacial skeleton and dentition (MAXWELL *et al.*, 1979), with differences in age (VIGNON *et al.*, 1980), or with topographical differences within muscles (MAXWELL *et al.*, 1979). However, interspecific variation reasonably may be due to differences in feeding specialisations among mammals.

Based on the staining intensitiy for myosin ATPase, NADH-TR and α-GPD, three histochemical fibre types are identified in the masticatory muscles of *Pteropus*. Each of these types exhibit similar characteristics as those reported in other mammalian masticatory and limb muscles. Accordingly, they were here designated as slow-twitch oxidative (SO), fast twitch oxidative glycolytic (FOG) and fast-twitch glycolytic (FG) (PETER *et al.*, 1972). The myosin ATPase activity is proportional to the intrinsic speed of contraction (BARANY, 1976). Two types of skeletal myosin have been described, which differ in ATPase activity. Myosin having high ATPase activity is alkaline-stable and acid-labile and dominates in fast muscle fibres. Myosin having low ATPase activity is acid-stable and alkaline-labile and predominates in slow muscle fibers (BURKE *et al.*, 1971; GUTH and SAMAHA, 1969; MAXWELL *et al.*, 1982; PETER *et al.*, 1972). The staining intensity for NADH-TR is indicative of the oxidative capacity of fibres and correlates with resistance to fatigue (BURKE *et al.*, 1971; PETER *et al.*, 1972). The intensity for α-GPD is a measure of the glycolytic capacity of fibres (PETTE and BÜCHER, 1963). In accordance with physiological and histochemical properties of single motor units (BURKE

et al., 1973), it appears that FG fibres are designed for rapid, powerful, but unsustained contractions. FOG fibres produce a smaller amount of tension, but are more fatigue resistant. SO fibres are very slow, produce the smallest tension, but are very resistant to fatigue.

Based upon relative proportions of fibre types, three muscle groups could be distinguished among the masticatory muscles of *Pteropus*. However, in each of these groups the fast-twitch muscle fibres dominate; all adductors contain a high percentage of fast-twitch fibres, ranging between 70-80 % in the masseter, but being greater than 90 % in the largest part of the temporalis. Pteropids are rather voracious animals that feed on soft fruit pulp and fruit juices. In *Pteropus* the chewing rate is 1-2.5 cycles per second (DE GUELDRE and DE VREE, 1984). Comparison with data on the masticatory muscles of other mammals suggests that the proportion of fast fibres in the main adductors is related to chewing rate. The latter is clearly a function of the masticated food, although body size may have some importance too. The masseter muscle of guinea pigs, rats and mice, which is their most important adductor, is composed entirely of fast-twitch fibres (MASUDA *et al.*, 1974; SCHIAFFINO, 1974; SUZUKI, 1977); chewing rates observed are respectively 6/second in guinea pigs (DE VREE, 1977) and 5-7/second in rats (THOMAS and PEYTON, 1983). In cats over 90 % of the fibres of their main adductor, the temporalis, and of the masseter, are fast-twitch (GORNIAK, 1986; TAYLOR *et al.*, 1973). As compared to guinea pigs and rats, their chewing rate is slower, being 3-3.5/second (GANS and GORNIAK, 1980). In humans (RINGQVIST, 1974; VIGNON *et al.*, 1980), macaques (CLARK and LUSCHEI, 1981), and pigs (HERRING *et al.*, 1979; SUZUKI, 1977) the masseter contains 60-80 % of fast-twitch fibres (exception : MAXWELL *et al.*, 1979); the available data for the temporalis show somewhat higher values. The chewing rate amounts 2.5-3.3/second in macaques (LUSCHEI and GOODWIN, 1974) and 3/second in pigs (HERRING and SCAPINO, 1973). In contrast, cattle and sheep masseter only contain slow-twitch fibres (SUZUKI, 1971, 1977; SUZUKI and TAMATE, 1974). Accordingly, their chewing rate is relativeley slow (40-70/minute, SUZUKI, 1977).

Muscles are not only designed to produce movement; they are also able to produce large forces, which is especially important in masticating food. Requirements for speed and power do not necessarily exclude each other. Physiological evidence suggests that muscles containing a high proportion of FG fibres mostly combine these functions. In the human masseter muscle a positive correlation is found between the area of FG fibres and maximal isometric bite force (RINGQVIST, 1974). However, FG fibres are not fatigue resistant. A masticatory muscle with a high percentage of SO and FOG fibres is more likely adapted to frequent and long activity. This explains why the masseter of guinea pigs, rats and mice is composed entirely of fast-twitch oxidative glycolytic fibres. A possible loss of tension production is compensated for by an increase of muscle mass. In cattle and sheep, a long and sustained action, but less force, is required for mastication and rumination. Hence, the reported fibre composition (only slow-twitch fibres), as well as mass of their masseter entirely fits these demands.

The masticatory muscles of *Pteropus* appear to be adapted to both speed and power. Speed is important after ingestion, when pieces of fruit are initially punctured by rapid, vigorous orthal movements of the lower jaw. On the other hand, power is required during the last bites of a reduction sequence, when the skin of the fruit pieces is forcefully crushed (De Gueldre and De Vree, 1984). The temporalis of *Pteropus* (histochemical group 1, over 90 % fast-twitch fibres) is the largest adductor, producing most of the bite force (De Gueldre and De Vree, 1988, 1990). Electromyography has revealed that its activity shows little variation in amplitude during the course of a sequence, as well as among food types. Electromyography also indicates that its main part is only active for a short period during the closing phase. Hence, the dominance of FG fibres in the superficial and medial parts of the temporalis may be correlated with requirements for both speed and power. In the cat, a positive correlation between the level and duration of EMG and the percentage of SO + FOG fibres, and a negative correlation with the percentage of FG fibres and the ratio of FG/ FOG + SO fibres has been demonstrated (Gorniak, 1986). The masseter and medial pterygoid muscles of *Pteropus* (group 2, 20-30 % of slow-twitch fibres) are generally active during diffent phases of the masticatory cycle (De Gueldre and De Vree, 1988). Furthermore, their activity changes considerably as a function of the position of the cycle in the reduction sequence and as a function of food consistency. Probably, their main function is to add chewing force when necessary, especially early and late in the reduction sequence, as well as in the early part of opening. The openers of *Pteropus* (group 3 muscles) contain high proportions of SO fibres (30-50 %). They are active throughout the main part of the chewing cycle, and are probably of little use in the production of force. Furthermore, the inverted position during feeding may account for their fibre composition.

Contraction characteristics

Some physiological parameters of muscle activity were obtained from muscles representative of two histochemical muscle groups. Since the tension was measured as closing force exerted by the mandible, the technique failed to give reliable results for the group 3 muscles. Hence, only the results from group 1 and group 2 muscles were presented.

The contraction characteristics obtained by in vivo stimulation of whole muscle are supposed to be representative of the behaviour of the muscle as a whole (Thexton and Hiiemae, 1975), especially in the range of the intensity of the applied stimuli (Nordstrom and Yemm, 1974), and to produce the same pattern of length-tension curve as does direct stimulation via the nerve (Mackenna and Türker, 1978). The first two premises are likely to be valid for *Pteropus,* since electromyography has shown that in this animal all muscle subdivisions considered behave homogeneously during function (De Gueldre and De Vree, 1988). The results thus may be assumed to represent at least those of the predominant fibres.

The contraction properties of the jaw muscles of *Pteropus,* as well as of other mammals for which data are available, appear to correlate with the histochemical

fibre composition. In *Pteropus* the temporalis is faster than the masseter and zygomaticomandibularis, but fatigues more rapidly during tetanic contractions. In opossums (THEXTON and HIIEMAE, 1975), rats (NORDSTROM and YEMM, 1974), guinea pigs (MASUDA *et al.*, 1974), cats (MACKENNA and TÜRKER, 1978 ; TAYLOR *et al.*, 1973), and macaques (MATSUNAMI and KUBOTA, 1972) the masseter, which is shown to contain more fast-twitch fibres, generally shows shorter twitch times as compared to *Pteropus*. In opossums and guinea pigs the temporalis is slower than the masseter, in cats it is faster. Tetanus frequency in the jaw muscles of *Pteropus* appears to be lower than in the rat masseter (NORDSTROM and YEMM, 1974). This is consistent with the fact that fast muscles show higher fusion frequency for tetani.

This study indicates that in the jaw closers of *Pteropus* maximum tension is developed when maximum gape is approached. Hence, our results agree with those of ANAPOL and HERRING (1989), MACKENNA and TÜRKER (1978), NORDSTROM and YEMM (1974), and THEXTON and HIIEMAE (1975). In the mouth openers, which we failed to study, maximum tension is reported to be developed with near-closed mouths (ANAPOL and HERRING, 1989 ; ANAPOL *et al.*, 1987 ; MACKENNA and TÜRKER, 1978). The only exception thus far is the optimum gape of 23° reported for the digastric muscle of the opossum (THEXTON and HIIEMAE, 1975). There are also indications that the tension produced during lengthening is slightly larger, than during subsequent shortening in the muscles studied. This effect was also reported in the opossum (THEXTON and HIIEMAE, 1975).

According to the sliding-filament theory the force-producing capacity of muscles is a function of sarcomere length (GORDON *et al.*, 1966). The effect of jaw opening on tension observed in whole jaw muscles may be related to changes in sarcomere length. Measurements of sarcomere length of masticatory muscles at different jaw positions confirm that in the adductors sarcomere length is significantly larger with the mouth open as compared to the closed position (HERRING *et al.*, 1979, 1984 ; HERZBERG *et al.*, 1980 ; MUHL *et al.*, 1978 ; NORDSTROM and YEMM, 1972 ; NORDSTROM *et al.*, 1974 ; WEIJS and VAN DER WIELEN-DRENT, 1983). In the abductors (digastric), sarcomere length is longer with the mouth closed (HERZBERG *et al.*, 1980). The longer sarcomere lengths are near the top of the ascending limb of the isometric length-tension relation (HERZBERG *et al.*, 1980 ; MUHL *et al.*, 1978). Hence, study of tension production on whole muscles is directly related to changes in sarcomere lengths. However, these studies also reveal regional variation in the effect of jaw position on sarcomere length within certain muscles (masseter) (HERRING *et al.*, 1979 ; HERZBERG *et al.*, 1980 ; NORDSTROM *et al.*, 1974 ; WEIJS and VAN DER WIELEN-DRENT, 1983). The assumption that not all fibres of a muscle reach their optimum at the same gape, concluded from the broad mean length-tension curve in certain masticatory muscles of the opossum (THEXTON and HIIEMAE, 1975), is consistent with these findings. However, this effect is probably only slightly present in the muscle subdivisions of *Pteropus*.

A functional interpretation of the length-tension relationships in the adductors of *Pteropus* is difficult. The superficial masseter and medial temporalis muscles are both effective in the nearly orthal closure of the mouth during mastication ; the zygomaticomandibularis muscle aids in the slight lateral deviation of the mandible

early in closure (De Gueldre and De Vree, 1988). Electromyograms obtained during normal feeding indicate that the muscles studied are not (superficial masseter muscle) or only slightly (medial temporalis and zygomaticomandibularis muscles) active at the optimum length measured in this study ; on the contrary, in all muscles maximum activity is produced with the jaws nearly closed (De Gueldre and De Vree, 1988). Only during biting are the muscles active with the jaws open. On the other hand, moments around the bicondylar or vertical (the latter mainly for the zygomaticomandibularis muscle) axis increase during closure in all three muscles (De Gueldre and De Vree, 1990). So it is possible that the optimum muscle lengths near maximum gape compensate for a decreased mechanical efficiency. In the digastric muscle moments around the bicondylar axis are indeed largest with the jaws open.

Conclusions

It appears that jaw muscles are able to regulate their fibre composition in response to the need for a certain chewing rate and force. Basic chewing rates observed among mammals are correlated with fibre distribution. Coincidently, variation in amplitude of EMG may represent a form of load compensation. In *Pteropus* this regulation seems to be muscle dependent (De Gueldre and De Vree, 1988, 1990). Jaw muscles with a higher proportion of oxidative fibres seem to be capable to regulate their muscular activity to a larger extent. Furthermore, sarcomere number in jaw muscles is adapted to allow the optimum sarcomere length to be reached at a certain gape (Weijs and Van der Wielen-Drent, 1983). Possibly, this sarcomere number is regulated by active tensions at jaw positions where the muscle is highly active (Active Position Hypothesis, Herring *et al.*, 1984), but muscle mechanics are probably involved also. Since it has been demonstrated that muscle fibre types reflect functional differences of the innervating motoneuron (Henneman and Olson, 1965 ; Yellin, 1967), it may be suggested that the structural and functional heterogeneity of the masticatory apparatus among mammals reflects differences in neural control.

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LIMB PROPORTIONS IN TERRESTRIAL MAMMALS

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SUMMARY

A large sample of terrestrial mammals (Primates excluded) was used to investigate the relative proportions between the lengths of the long bones. In a more reduced number of species, regressions between limb length against body mass, diameters of proximal and distal bones, and metapodial lengths, were calculated. There appears a strong tendency towards isometry, in cases where lengths or diameters are compared. The limb length scales against body mass with an exponent close to that previously postulated (0.34).

Keywords : Limb bones, proportions, mammals, limb length.

INTRODUCTION

Controversies about the exact explanation of the scaling of the skeleton have produced a very important amount of literature on the relationships between diameter and length of the long bones in different groups of mammals and of each one of those parameters to body mass, namely, dogs (CASINOS *et al.* 1986), insectivores and rodents (BOU *et al.* 1987), primates (AIELLO 1981), ungulates (ALEXANDER 1977 ; MCMAHON 1975), generality of mammals (ALEXANDER *et al.* 1979). Nevertheless, other kind of relationships have been almost untouched. For example, only AIELLO (1981) refers to the relative length of the limb bones in primates.

This paper tries to show a comprehensive view of the proportions between the length of the bones of the fore and hindlimbs (including metapodials) and among their diameters in a large sample of terrestrial mammals. In two special cases (insectivores and rodents) body masses were available and the analysis is more extensive.

Consider a strictly quadrupedal mammal. In principle, we may imagine it as an animal with a horizontal vertebral column supported by both girdles and the two

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pairs of limbs. For that kind of mammal, we formulated the following hypotheses to be tested :

1) In both the fore and hind legs, the proximal long bone scales against the distal long bone in an isometric way.

2) Any bone of the forelimb (proximal, distal or the metapodial) is directly proportional to its homologue of the hind limb.

3) Humerus + ulna must be directly proportional to femur + tibia.

4) We may suppose that stresses on midshaft of the proximal bone of each limb equal stresses on the same place in the distal bone. In this case, and taking into account that, according to ALEXANDER (1983), the bending moment on a beam is proportional to the second moment of the area, the latter being proportional to the radius, the regression between the midshaft anteroposterior diameters of both the proximal and distal bones must be isometric.

5) If animals are geometrically similar, any linear dimension must scale with the cube root of body mass. Therefore, the addition of both the long bones of a leg (humerus + ulna or femur + tibia) have to be proportional to $M^{0.33}$. Nevertheless, ALEXANDER *et al.* (1979) found an exponent value of 0.34.

In our sample, not strictly quadrupedal mammals were also included. The idea was to see in which way a particular type of locomotion could modify the results obtained with quadrupedal animals.

HOWELL (1944) carried out a similar kind of research, from a generic point of view, but his data were treated as indices and nothing about scaling can be concluded. Moreover, as AIELLO (1981) says, the use of indices is only correct when both variables comprising it change isometrically.

MATERIAL AND METHODS

The leg bone length of 396 specimens of terrestrial mammals was measured. They were from 119 different species, belonging to 13 orders. The body mass of 96 of those specimens (25 species) was known. All of them were insectivores or rodents. The anteroposterior diameter, at midshaft of the long bones, was available from 236 specimens (36 species). Finally, the length of the longest metacarpal and metatarsal in 74 specimens (31 species) was used. The species were :

O. Monotremata
  F. Tachyglossidae
    Tachyglossus aculeatus (SHAW and NODDER, 1792) (1)
  F. Ornithorhynchidae
    Ornithorhynchus anatinus (SHAW and NODDER, 1799) (1)

O. Marsupialia
  F. Didelphidae
    Didelphis marsupialis LINNAEUS, 1758 (1)

F. Thylaccinidae
Thylacinus cynocephalus (HARRIS, 1808) (1)
F. Notoryctidae
Notoryctes typhlops (STIRLING, 1889) (1)
F. Phascolarctidae
Phascolarctos cinereus (GOLDFUSS, 1817) (1)
F. Vombatidae
Vombatus ursinus (SHAW, 1800) (1)

O. Edentata
F. Myrmecophagidae
Myrmecophaga tridactyla LINNAEUS, 1758 (1)
F. Bradypodidae
Bradypus tridactylus LINNAEUS, 1758 (1)

O. Insectivora
F. Chrysocloridae
Chrysospalax trevelyani (GUNTHER, 1875) (3)
Chrysocloris sp. LACÉPÈDE, 1799 (3)
F. Erinacidae
Erinaceus sp. LINNAEUS, 1758 (5)
F. Soricidae
Crocidura russula (HERMANN, 1780) (5)
Sorex araneus LINNAEUS, 1758 (5)
Sorex minutus LINNAEUS, 1766 (4)
Suncus etruscus (SAVI, 1822) (2)
F. Talpidae
Galemys pyrenaicus (GEOFFROY, 1811) (4)
Talpa europaea LINNAEUS, 1758 (5)

O. Carnivora
F. Canidae
Canis lupus LINNAEUS, 1758 (12)
Canis lupus (familiaris) LINNAEUS, 1758 (149)
Canis mesomelas SCHREBER, 1778 (1)
Vulpes vulpes (LINNAEUS, 1758) (1)
F. Ursidae
Ursus americanus PALLAS, 1780 (1)
Ursus arctos LINNAEUS, 1758 (2)
F. Mustelidae
Mustela putorius LINNAEUS, 1758 (1)
F. Viverridae
Genetta genetta (LINNAEUS, 1758) (1)
F. Herpestidae
Ichneumia albicauda (CUVIER, 1829) (1)

F. Hyaenidae
Crocuta crocuta (ERXLEBEN, 1777) (1)
F. Felidae
Acinonyx jubatus (SCHREBER, 1776) (1)
Felis silvestris SCHREBER, 1777 (1)
Lynx lynx (LINNAEUS, 1758) (1)
Panthera leo (LINNAEUS, 1758) (3)
Panthera pardus (LINNAEUS, 1758) (1)
Panthera tigris (LINNAEUS, 1758) (1)

O. Proboscidea
F. Elephantidae
Elephas maximus LINNAEUS, 1758 (2)
Loxodonta africana (BLUMEMBACH, 1797) (3)

O. Perissodactyla
F. Equidae
Equus caballus LINNAEUS, 1758 (2)
Equus hemionus PALLAS, 1775 (1)
Equus grevyi OUSTALET, 1882 (1)
Equus quagga GMELIN, 1788 (1)
F. Tapiridae
Tapirus indicus DESMAREST, 1819 (1)
F. Rhinocerontidae
Diceros bicornis (LINNAEUS, 1758) (1)

O. Tubulidentata
F. Orycteropodidae
Orycteropus afer (PALLAS, 1766) (1)

O. Artiodactyla
F. Suidae
Phacochoerus aethiiopicus (PALLAS, 1767) (2)
Potamochoerus porcus (LINNAEUS, 1758) (1)
Sus salvanius (HODGSON, 1847) (1)
Suc scrofa LINNAEUS, 1758 (1)
F. Tayassuidae
Tayassu tajacu (LINNAEUS, 1758) (1)
F. Hippopotamidae
Hippopotamus amphibius LINNAEUS, 1758 (1)
F. Camelidae
Camelus dromedarius LINNAEUS, 1758 (1)
Camelus bactrianus LINNAEUS, 1758 (1)
F. Cervidae
Alces sp. Gray, 1821 (1)
Capreolus capreolus (LINNAEUS, 1758) (1)

Cervus dama LINNAEUS, 1758 (1)
Cervus elaphus LINNAEUS, 1758 (1)
Rangifer tarandus (LINNAEUS, 1758) (1)

F. Giraffidae
Giraffa camelopardalis (LINNAEUS, 1758) (1)

F. Bovidae
Addax nasomaculatus (BLAINVILLE, 1816) (1)
Aepyceros melampus (LICHTENSTEIN, 1812) (1)
Antidorcas sp. SUNDEVALL, 1847 (1)
Bison bison (LINNAEUS, 1758) (1)
Bos frontalis LAMBERT, 1804 (1)
Bos javanicus D'ALTON, 1823 (1)
Boselaphus tragocamelus (PALLAS, 1766) (1)
Bubalus bubalis (LINNAEUS, 1758) (1)
Bubalus depressicornis (H. SMITH, 1827) (1)
Capra hircus (LINNAEUS, 1758) (1)
Capra ibex LINNAEUS, 1758 (1)
Connochaetes gnou (ZIMMERMANN, 1780) (2)
Connochaetes taurinus (BURCHELL, 1824) (1)
Gazella granti BROOKE, 1872 (2)
Gazella thomsoni GUNTHER, 1884 (1)
Hippotragus equinus (DESMAREST, 1804) (1)
Hippotragus niger (HARRIS, 1838) (1)
Kobus ellipsiprymus (OGILBY, 1833) (1)
Kobus kob (ERXLEBEN, 1777) (1)
Litrocanius walleri (BROOKE, 1879) (1)
Madoqua kirki (GUNTHER, 1880) (2)
Oryx dammah (CRETZSCHMAR, 1826) (1)
Oryx gazella (LINNAEUS, 1758) (1)
Ovis aries LINNAEUS, 1758 (1)
Rupicapra rupicapra (LINNAEUS, 1758) (1)
Saiga sp. GRAY, 1843 (1)
Syncerus caffer (SPARRMAN, 1779) (1)

O. Pholidota

F. Manidae
Manis tricuspis RAFINESQUE, 1821 (1)

O. Rodentia

F. Sciuridae
Cynomys sp. RAFINESQUE, 1817 (2)
Iomys horsfieldii (WATERHOUSE, 1838) (1)
Marmota marmota (LINNAEUS, 1758) (5)
Petinomis vordermanni (JENTIK, 1890) (4)
Sciurus vulgaris LINNAEUS, 1758 (2)
Spermophilopsis leptadactylus (LICHTENSTEIN, 1823) (1)

F. Geomydae
Geomys sp. RAFINESQUE, 1817 (1)

F. Castoridae
Castor canadiensis KUHL, 1820 (2)
Castor fiber LINNAEUS, 1758 (2)

F. Pedetidae
Pedetes capensis (FORSTER, 1778) (1)

F. Cricetidae
Cricetomys gambianus WATERHOUSE, 1840 (5)
Meriones sacramenti THOMAS, 1922 (5)
Mesocricetus auratus (WATERHOUSE, 1839) (5)

F. Spalacidae
Spalax sp. GUNDENSTAEDT, 1770 (5)

F. Rhizomydae
Rhizomys sp. GRAY, 1831 (2)

F. Arvicolidae
Arvicola sapidus MILLER, 1908 (5)
Arvicola terrestris (LINNAEUS, 1758) (5)
Clethrionomys sp. TILESIUS, 1850 (5)
Pitymis duodecimcostatus (S-LONGCHAMPS, 1839) (5)

F. Muridae
Acomys cahirinus (DESMAREST, 1891) (1)
Apodemus sylvaticus (LINNAEUS, 1758) (1)
Mus musculus LINNAEUS, 1766 (5)
Rattus norvegicus (BERKENHOUT, 1769) (5)
Rattus rattus (LINNAEUS, 1758) (5)

F. Gliridae
Eliomys quercinus (LINNAEUS, 1766) (5)
Myoxus glis LINNAEUS, 1766 (3)

F. Caviidae
Cavia porcellus (LINNAEUS, 1758) (5)
Dolichotes patagonum (ZIMMERMANN, 1780) (4)

F. Hydrochaeridae
Hydrochaeris hydrochaeris (LINNAEUS, 1766) (1)

F. Dasyproctidae
Dasyprocta leporina (LINNAEUS, 1758) (3)
Myoprocta acouchy (ERXLEBEN, 1777) (4)

O. Lagomorpha

F. Leporidae
Lepus capensis LINNAEUS, 1758 (1)
Oryctolagus cuniculus (LINNAEUS, 1758) (2)

O. Macroscelida
F. Macroscelididae
Rhynchocyon chrysopygus GUNTHER, 1881 (1)

The data on insectivores and rodents and dog breeds had already been used in other researches (BOU *et al.* 1987 ; CASINOS *et al.* 1986). See this second paper for a detailed list of the dog breeds. The name of every species is followed by the number of specimens studied in parentheses.

Vernier callipers were used for measuring. The length was always the functional one (i.e., the distance between the middle points of the two opposite articulations). Regression coefficients were calculated by means of Model II or reduced major axis method. When comparisons with theoretical exponents were required, Student t-tests were used. For more details about the mathematical methodology see BOU *et al.* (1987). Separate allometric equations were calculated for the different orders, namely Marsupialia, Insectivora, Carnivora, Proboscidea, Perissodactyla, Artiodactyla and Rodentia. Moreover, for Carnivora a separate equation was calculated for the genus *Canis,* because of the large size of the sample. For Monotremata, Edentata, Tubulidentata, Pholidota and Lagomorpha, individual equations were not calculated because the minimum number of five specimens, considered indispensable for calculations, was not available. Those data were only used for calculations of general correlations of all mammals. The only big major order that was not studied was that of Primates. Results of AIELLO (1981) were considered sufficient.

According to the hypotheses assumed (see above), the following regressions were estimated : length of the humerus to length of the ulna ; length of the femur to length of the tibia (in both cases the independent variable was always the proximal bone) ; length of the femur to length of the humerus ; length of the tibia to length of the ulna ; length of femur + tibia to length of humerus + ulna ; length of the longest metatarsal to length of the longest metacarpian (in those cases the hindlimb bone (or bones) was the independent variable) ; body mass to length of humerus + ulna, and femur + tibia (body mass as independent variable) ; diameter of the humerus to diameter of the ulna ; diameter of the femur to diameter of the tibia (the diameter of the proximal bone was taken in all cases as independent variable).

RESULTS

Results are given separately for each kind of regression.

Relative proportions between the forelimb long bones. — Separate equations were calculated for all the mammal groups quoted above and the whole sample. They are shown in Table 1. Taking into account that according to our first hypothesis the isometric condition was considered the most logical in a perfect quadrupedal mammal, the exponents of the equation were compared to the theoretical slope of 1. In three cases (Marsupialia, *Canis* sp., Artiodactyla and all mammals) the calculated exponents differ significantly from 1. The only case in

which the correlation coefficient is not really high is in the order Perissodactyla. The dispersion of values is caused by the separation between the Equidae and the other two species (*Tapirus indicus* (TI) and *Diceros bicornis* (DB)) (Fig. 1).

TABLE 1

Allometric equations for the relationship between humerus and ulna. r, *correlation coefficient ;* n, *number of specimens ; D and E, mean « different » and « equal » to the theoretical exponent, respectively.*

		r	*n*	
Marsupialia	$y = 0.66 x^{1.19}$	0.997	5	D P < 0.05
Insectivora	$y = 1.01 x^{0.89}$	0.942	36	E P > 0.05
Carnivora	$y = 1.07 x^{0.98}$	0.988	20	E P > 0.05
Canis sp.	$y = 0.74 x^{1.12}$	0.989	158	D P < 0.05
Proboscidea	$y = 0.40 x^{1.15}$	0.982	5	E P > 0.05
Perissodactyla	$y = 1.36 x^{0.95}$	0.678	8	E P > 0.05
Artiodactyla	$y = 0.67 x^{1.19}$	0.893	45	D P < 0.05
Rodentia	$y = 0.99 x^{1.02}$	0.984	110	E P > 0.05
Mammalia	$y = 0.98 x^{1.02}$	0.993	396	D P < 0.05

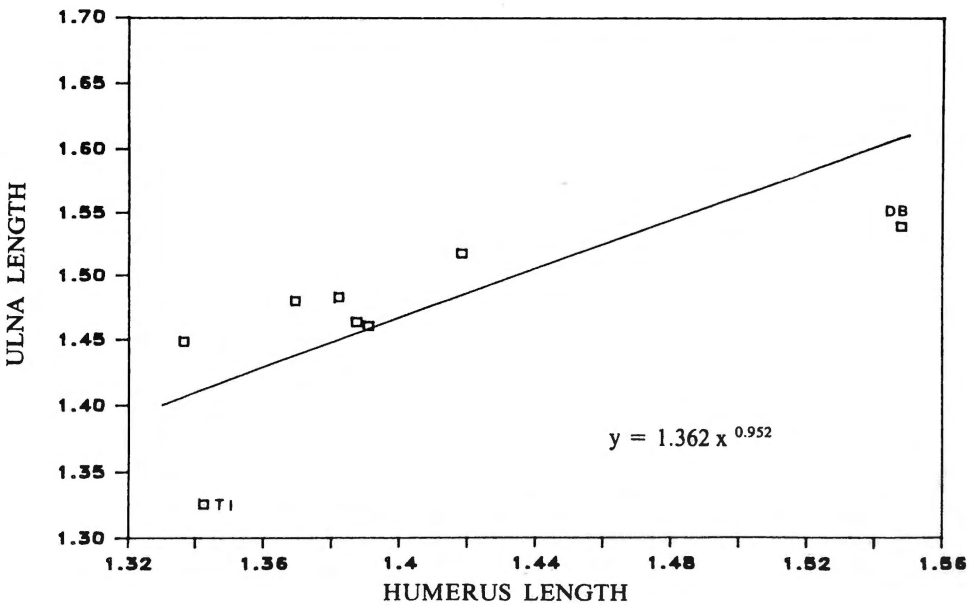

Fig. 1. — Graph of logarithmic coordinates of ulnar length against humeral length for Perissodactyla. DB, *Diceros bicornis* ; TI, *Tapirus indicus*.

Relative proportions between the hindlimb long bones. — In Table 2 the equations calculated for the different groups and for the whole sample are shown. In four cases (Insectivora, Carnivora, *Canis* sp. and the general regression) the achieved slopes are different from 1. Again the Perissodactyla have the lowest correlation coefficient. Their points are distributed in a similar way to that which we have seen before.

TABLE 2

Allometric equations for the relationship between femur and tibia.
See table 1 for abbreviations.

		r	*n*	
Marsupialia	$y = 0.84 x^{1.02}$	0.992	5	E P > 0.05
Insectivora	$y = 1.43 x^{0.82}$	0.889	36	D P < 0.05
Carnivora	$y = 1.49 x^{0.83}$	0.986	20	D P < 0.05
Canis sp.	$y = 0.91 x^{1.04}$	0.991	158	D P < 0.05
Proboscidea	$y = 0.57 x^{1.01}$	0.943	5	E P > 0.05
Perissodactyla	$y = 2.97 x^{0.66}$	0.729	8	E P > 0.05
Artiodactyla	$y = 1.42 x^{0.92}$	0.925	45	E P > 0.05
Rodentia	$y = 1.14 x^{1.02}$	0.987	110	E P > 0.05
Mammalia	$y = 1.29 x^{0.91}$	0.991	396	D P < 0.05

TABLE 3

Allometric equations for the relationship between humerus and femur.
Abbreviations as in table 1.

		r	*n*	
Marsupialia	$y = 0.94 x^{0.94}$	0.999	5	D P < 0.05
Insectivora	$y = 0.91 x^{1.05}$	0.991	36	D P < 0.05
Carnivora	$y = 0.94 x^{0.98}$	0.998	20	E P > 0.05
Canis sp.	$y = 1.05 x^{0.95}$	0.994	158	D P < 0.05
Proboscidea	$y = 0.40 x^{1.16}$	0.994	5	E P > 0.05
Perissodactyla	$y = 0.45 x^{1.15}$	0.974	8	E P > 0.05
Artiodactyla	$y = 0.73 x^{1.03}$	0.975	45	E P > 0.05
Rodentia	$y = 0.87 x^{1.01}$	0.989	110	E P > 0.05
Mammalia	$y = 0.87 x^{1.00}$	0.996	396	E P > 0.05

Relative proportions between the proximal long bones. — Only three of the nine equations show slopes different from the isometric condition (Marsupialia, Insectivora and *Canis sp.*) (Table 3) although in the first two cases the results of the Student-t tests are in the limit. In the general plot for all the studied mammals, values

from two species (*Pedetes capensis* (PE) and *Bradypus tridactylus* (BT)) are really distant from the general regression line (Fig. 2).

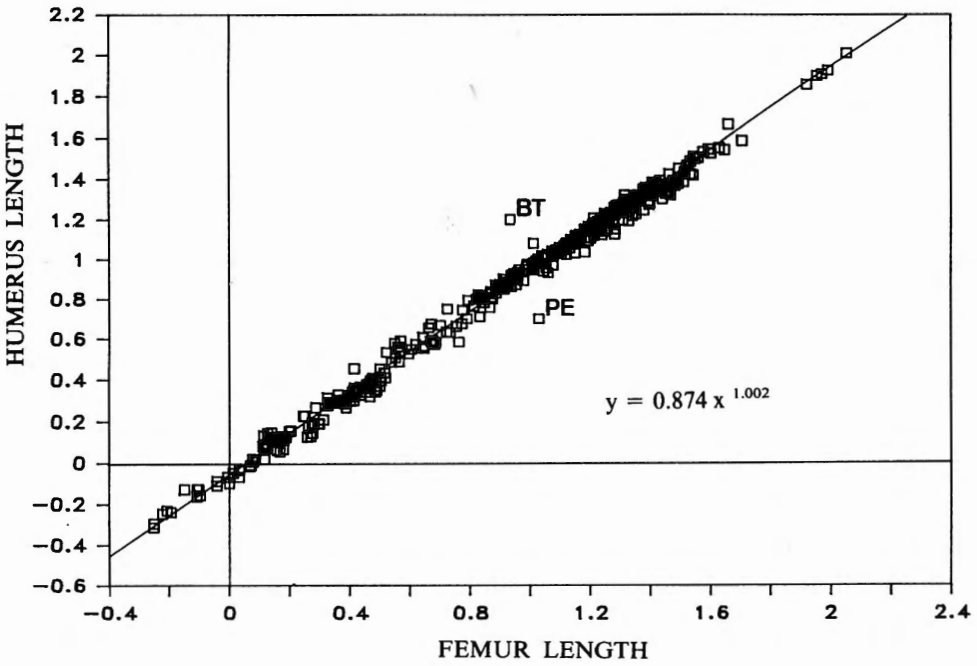

Fig. 2. — Graph of logarithmic coordinates of humeral length against femoral length for all the studied species. BT, *Bradypus tridactylus*; PE, *Pedetes capensis*.

TABLE 4

Allometric equations for the relationship between ulna and tibia.
Abbreviations as in table 1.

		r	*n*	
Marsupialia	$y = 0.74\ x^{1.09}$	0.989	5	E P > 0.05
Insectivora	$y = 0.61\ x^{1.14}$	0.977	36	D P < 0.05
Carnivora	$y = 0.64\ x^{1.14}$	0.986	20	D P < 0.05
Canis sp.	$y = 0.86\ x^{1.02}$	0.991	158	D P < 0.05
Proboscidea	$y = 0.29\ x^{1.32}$	0.927	5	E P > 0.05
Perissodactyla	$y = 0.10\ x^{1.65}$	0.978	8	D P < 0.05
Artiodactyla	$y = 0.29\ x^{1.33}$	0.961	45	D P < 0.05
Rodentia	$y = 0.71\ x^{1.01}$	0.983	110	E P > 0.05
Mammalia	$y = 0.64\ x^{1.12}$	0.944	396	D P < 0.05

Relative proportions between the distal long bones. — Only three of the exponents, those of Marsupialia, Proboscidea and Rodentia, are not significantly different from 1 (Table 4). The correlation coefficients are always very high. Again, in the general plot for all the mammals the values of *Pedetes capensis* and *Bradypus tridactylus* are placed below and above the regression line, respectively : this means that, while the former species has a relatively shorter ulna than the general tendency, that of the latter is longer.

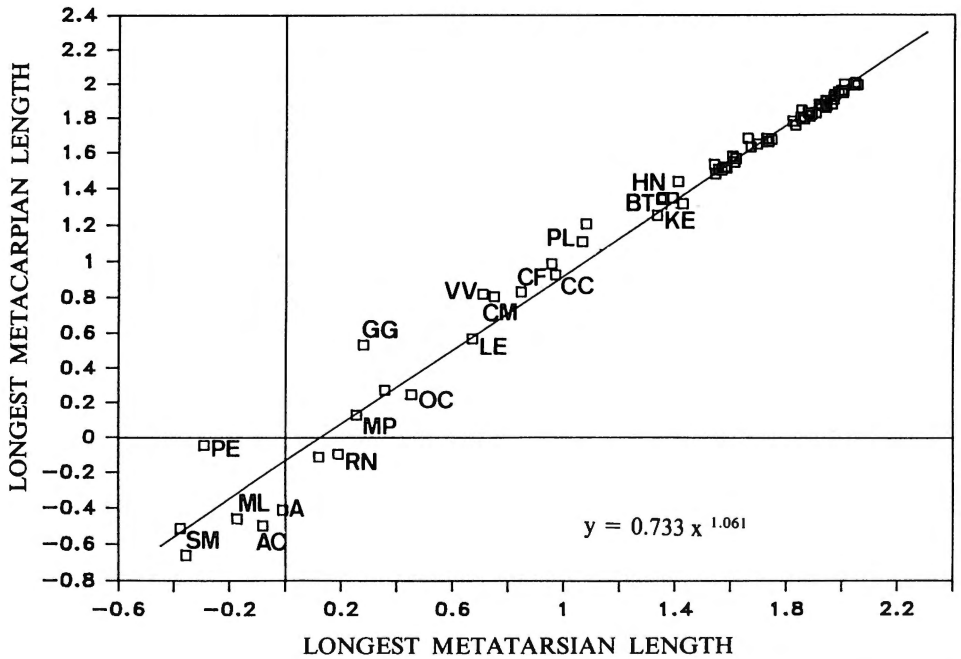

Fig. 3. — Graph of logarithmic coordinates of the longest metacarpian length against the longest metatarsian length for all the studied species.
Symbols other that used previously : A, *Apodemus sylvaticus* ; AC, *Acomys cahirinus* ; BT, *Boselaphus tragocamelus* ; CC, *Crocuta crocuta* ; CF, *Castor fiber* ; CM, *Canis mesomelas* ; GG, *Genetta genetta* ; HN, *Hippotragus niger* ; KE, *Kobus ellipsiprymus* ; LE, *Lepus capensis* ; ML, *Mus musculus* ; MP, *Mustela putorius* ; OC, *Oryctolagus cuniculus* ; PL, *Panthera leo* ; RN, *Rattus norvegicus* ; SM, *Sorex minutus* ; VV, *Vulpes vulpes*.

Relative proportions between among metapodials. — Only one equation was calculated- that of the whole sample (Table 5). Altough the achieved exponent (1.06) is very close to 1, they are statistically different. In figure 3 it is shown that the dispersion of values is particularly important in the smallest range of sizes, while for big body masses, values are remarkably united.

TABLE 5

Allometric equations for all the sample for the relationship between the longest metapodials. Abbreviations are the same that used in table 1.

		r	*n*
Insectivora			2
Carnivora (including *Canis* sp.)			52
Proboscidea			1
Artiodactyla			10
Rodentia			6
Lagomorpha			3
Mammalia	$y = 0.73 x^{1.06}$	0.991	74

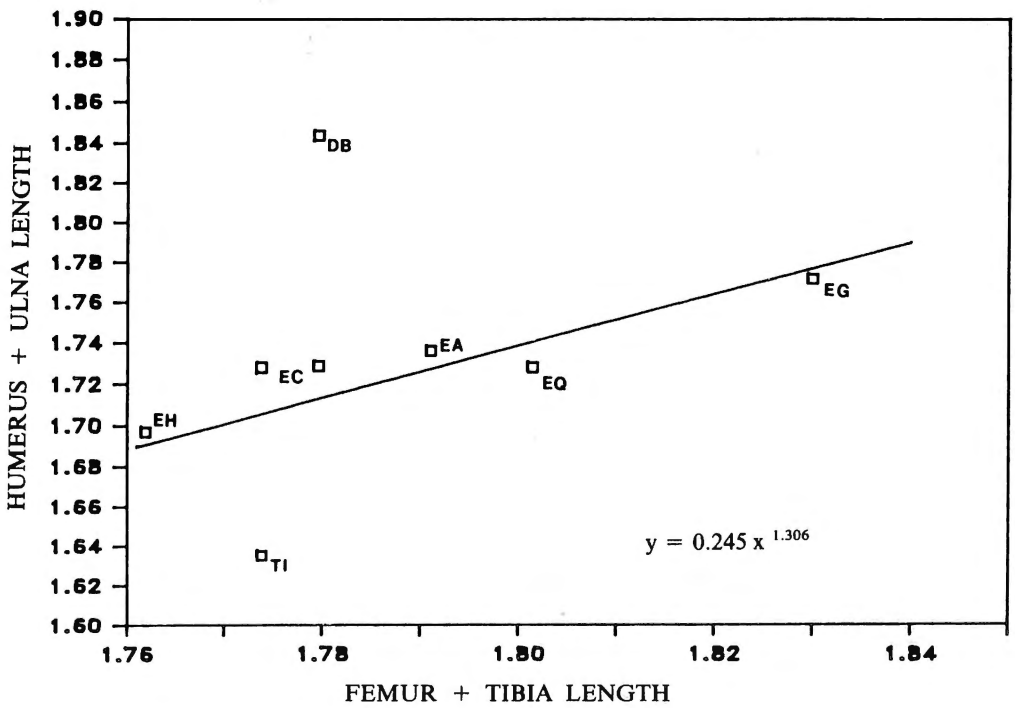

Fig. 4. — Graph of logarithmic coordinates of the forelimb (humerus + ulna) length against the hindlimb (femur + tibia) length for Perissodactyla.
Symbols other that used before : EA, *Equus asinus* ; EC, *Equus caballus* ; EH, *Equus hemionus* ; EG, *Equus grevyi* ; EQ, *Equus quagga*.

Relative proportions between the fore and hindlimbs. — Five of the slopes differ significantly from 1 ; other five can be considered equal (Table 6). The different

plots were remarkably uniform with the only exception of Perissodactyla (Fig. 4). Among the values of this order of mammals, *Diceros bicornis* (DB) has an extremly long forelimb (humerus + ulna) while that *Tapirus indicus* (TI) is rather short. The general regression line for all the sample appears very similar to that of figure 2, with the same relative positions as that of *Pedetes capensis* and *Bradypus tridactylus*.

TABLE 6

Allometric equations for the relationship humerus + ulna and femur + tibia.
See table 1 for abbreviations.

		r	*n*	
Marsupialia	$y = 0.82 x^{1.01}$	0.997	5	E P > 0.05
Insectivora	$y = 0.67 x^{1.12}$	0.991	36	D P < 0.05
Carnivora	$y = 0.76 x^{1.05}$	0.996	20	D P < 0.05
Canis sp.	$y = 0.96 x^{0.99}$	0.995	158	E P > 0.05
Proboscidea	$y = 0.27 x^{1.25}$	0.992	5	E P > 0.05
Perissodactyla	$y = 0.24 x^{1.30}$	0.972	8	D P < 0.05
Artiodactyla	$y = 0.41 x^{1.18}$	0.977	45	D P < 0.05
Rodentia	$y = 0.76 x^{1.01}$	0.988	110	E P > 0.05
Mammalia	$y = 0.71 x^{1.06}$	0.996	396	D P < 0.05

TABLE 7

Allometric equations obtained from the correlation of body mass against humerus + ulna or femur + tibia. F and H mean forelimb and hindlimb, respectively.
For other abbreviations, see table 1.

			r	*n*	
Insectivora	F	$y = 0.76 x^{0.32}$	0.960	25	E P > 0.05
	H	$y = 1.16 x^{0.28}$	0.973	25	D P < 0.05
Rodentia	F	$y = 0.78 x^{0.35}$	0.930	71	E P > 0.05
	H	$y = 1.02 x^{0.35}$	0.915	71	E P > 0.05

Scaling of the limb length to body mass. — The body mass was only available from insectivores and rodents. In this case the achieved exponents were tested against the theoretical value (0.34) postulated by ALEXANDER *et al.* (1979) (see above). Only the slope corresponding to the insectivore hindlimb is different from 0.34 (Table 7). The exceptions to the general tendency often appears as equal in both orders. That is to say, in insectivores *Talpa europea* (T) shows both legs shorter than expected (Fig. 5). Among rodents, the flying squirrels (*Iomys horsfieldii* (I),

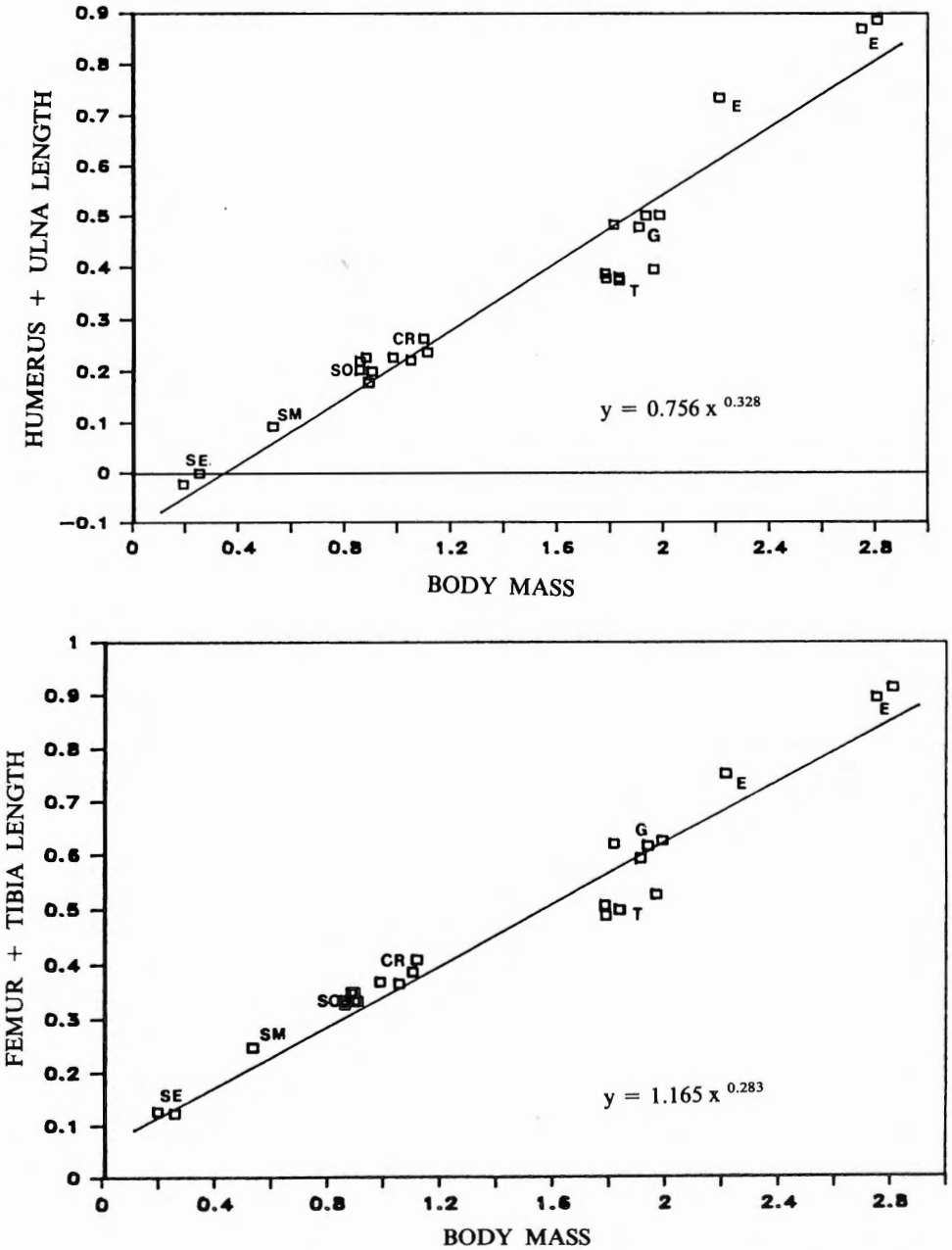

Fig. 5. — Graph of logarithmic coordinates of the forelimb (humerus + ulna) lenght against body mass (A) and of the hindlimb (femur + tibia) length against body mass (B), both for insectivores.

Symbols other that used above : CR, *Crocidura russula* ; E, *Erinaceus* sp. ; G, *Galemys pyrenaicus* ; SE, *Suncus etruscus* ; SM, *Sorex minutus* ; SO, *Sorex araneus* ; T, *Talpa europaea*.

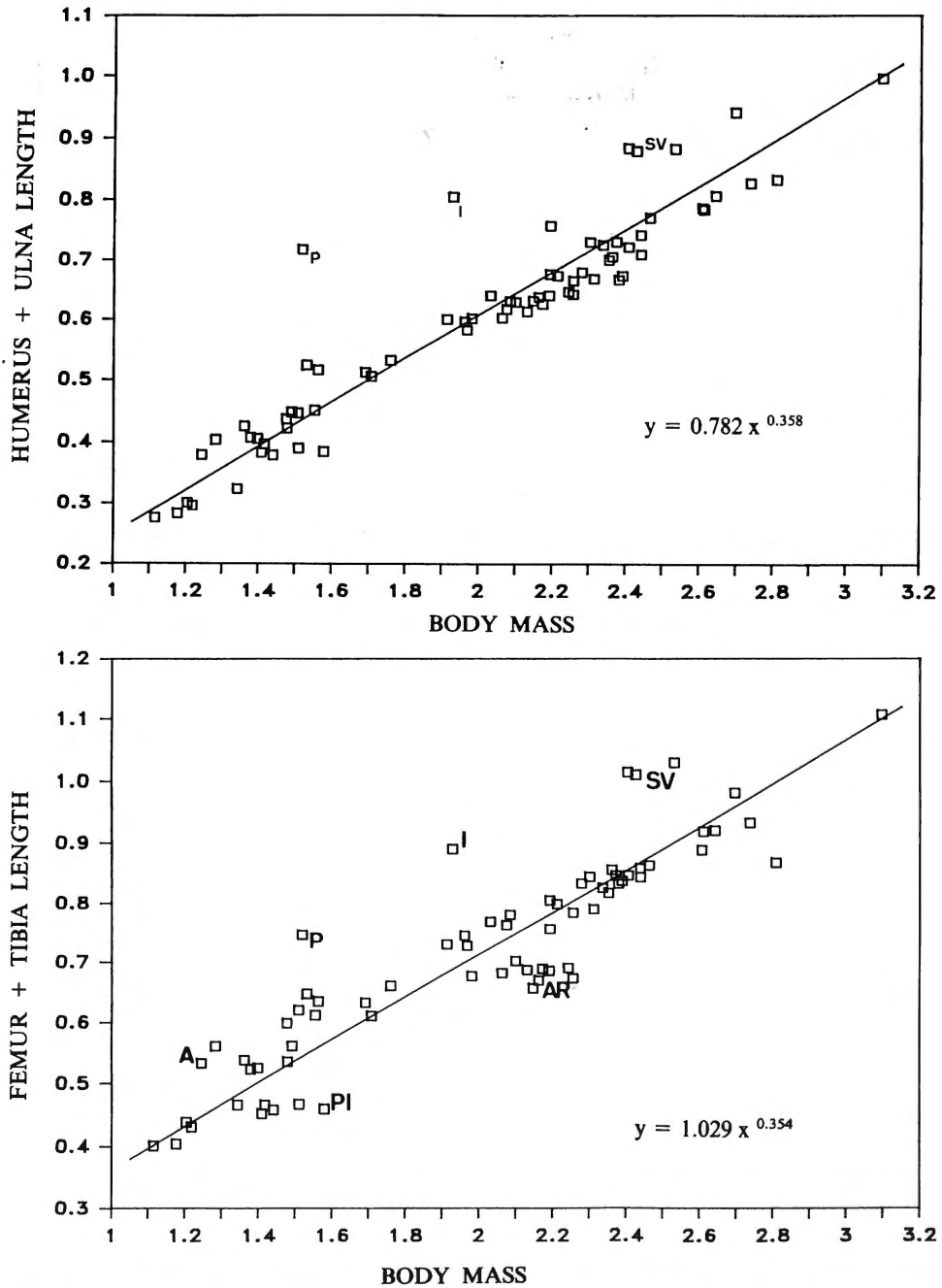

Fig. 6. — Graph of logarithmic coordinates of the forelimb (humerus + ulna) length against body mass (A) and of the hindlimb (femur + tibia) length against body mass (B), both for rodents.

Symbols not used before : AR, *Arvicola terrestris* ; I, *Iomys horsfieldii* ; P, *Petinomys vordermanni* ; PI, *Pitymys duodecimcostatus* ; SV, *Sciurus vulgaris*.

Petinomis vondermanni (P)) and *Sciurus vulgaris* (SV) are clearly above the regression lines of both legs, while *Pitymys duodecimcostatus* (PI) and *Arvicola terrestris* (AR) have all their values below the hindlimb regression line (Fig. 6). Similar results had been achieved previously for individual bones (BOU *et al.* 1987).

TABLE 8

Allometric equations achieved from the relationship between the diameters of the fore and hindlimb long bones.
Abbreviations as in table 7.

			r	*n*	
Insectivora	F	$y = 0.75 x^{0.86}$	0.975	30	D P < 0.05
	H	$y = 0.84 x^{0.99}$	0.939	30	E P > 0.05
Canis sp.	F	$y = 0.45 x^{1.04}$	0.864	135	E P > 0.05
	H	$y = 1.06 x^{0.96}$	0.957	135	E P > 0.05
Rodentia	F	$y = 0.85 x^{0.95}$	0.914	71	E P > 0.05
	H	$y = 0.83 x^{1.10}$	0.987	71	D P < 0.05

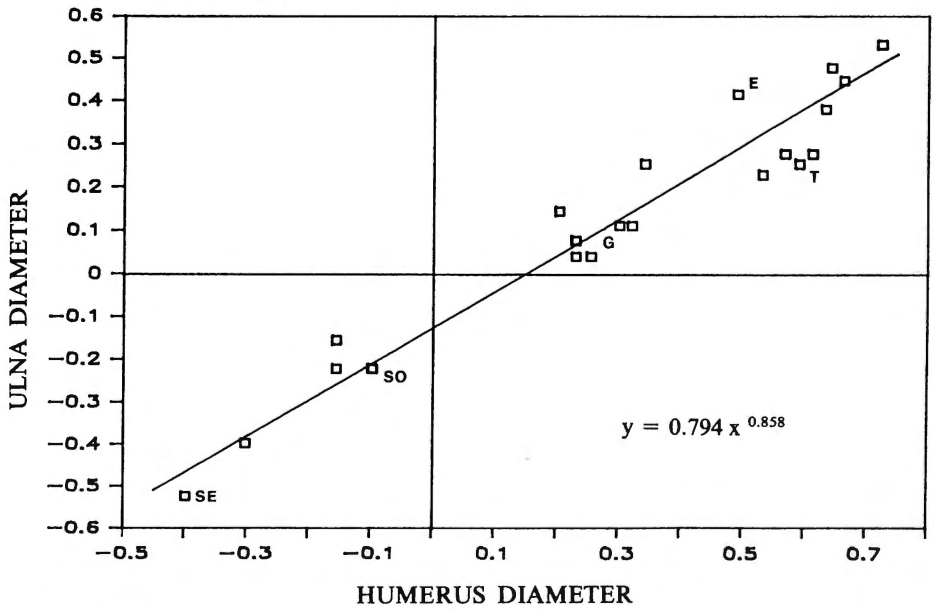

Fig. 7. — Graph of logarithmic coordinates of the ulnar diameter against humeral diameter for insectivores.

Relative proportions between diameters of the long bones. — Separated comparisons were established between the foreleg long bones (humerus + ulna) and the

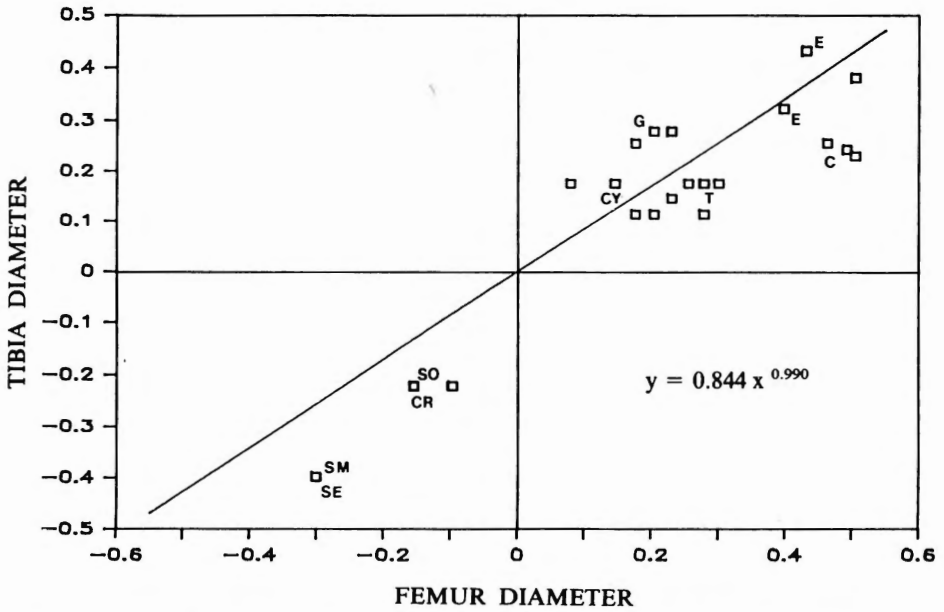

Fig. 8. — Graph of logarithmic coordinates of the tibial diameter against femoral diameter for insectivores.

Symbols not used before : C, *Chrysospalax trevelyani* ; CY, *Chrysocloris* sp.

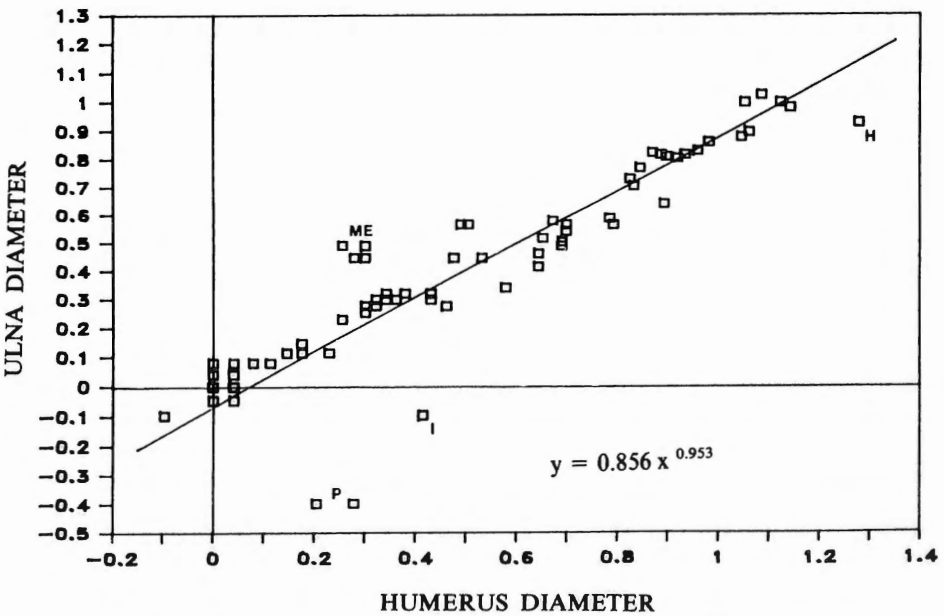

Fig. 9. — Graph of logarithmic coordinates of the ulnar diameter against humeral diameter for rodents.

Symbols not used previously : H, *Hydrochaeris hydrochaeris* ; ME, *Mesocricetus auratus*.

hindleg long bones (femur + tibia) for specimens of insectivores, *Canis sp.* and rodents. That is to say for the groups from which the diameters were available. The hypothetical condition, according to hypothesis number 4 was isometry. Table 8 shows that the exponents for the insectivore forelimb and the rodent hindlimb are significantly different from 1. In the first case the reason is possibly the position of the values of *Talpa europaea* (T), with a humerus wider than the ulna (Fig. 7). The small insectivores, like *Sorex araneus* (SO), *Crocidura russula* (Cr), *Sorex minutus* (SM) and *Suncus etruscus* (SE) have a remarkably slender tibia (Fig. 8). In a similar way, the flying squirrels *Petinomys vondermanni* (P) and *Iomys horsfieldii* (I) show a diameter decreasing from humerus to ulna (Fig. 9), which had been previously expected (BOU *et al.* 1987).

DISCUSSION

In general, in all the cases in which different lengths or diameters were compared, there is a tendency towards isometry. Species that appear far from the normal tendency show very particular kinds of locomotion, like *Pedetes capensis,* a very specialized jumping rodent (OFFERMANS and DE VREE 1988), with hindlegs very much longer than forelegs, and *Bradypus tridactylus* (Edentata), a typical arboreal species, in which case it is not surprising that it shows a reverse leg adaptation, according to previous results (BOU *et al.* 1987). For lengths, perhaps it is in Proboscidea, Perissodactyla and Artiodactyla where that tendency is less evident. In all the correlations calculated, one or more of those three orders shows the strongest positive or negative allometries. In Perissodactyla the positions of *Diceros bicornis* and *Tapirus indicus* are the most interesting. We have seen that the rhino has a relatively longer forelimb, while that of tapir is relatively short. But both species have shorter ulnae in comparison with the humeri. All this seems to indicate that the rhino has either a long humerus or a short hindlimb, according to its size, but unfortunately no data are available about this. The rhino has the head closer to the ground than the tapir, because of feeding habits, and that can mean a lowering of the center of mass. In general, it seems that the heavier the skull, the longer the forelimb is, taking into account the results of Proboscidea for the scaling of the forelimb against the hindlimb, which show a relatively important positive allometry (slope equals to 1.25 ; see Table 6). This can be an example that other functions than locomotion can exert their selective pressure on the limb design.

Because of the reduced size of the sample in Proboscidea and Perissodactyla, some mathematical artefact could be thought to be the cause of the allometries, but this is not the case in the Artiodactyla, where the number of studied specimens is very much more important. Thus, the different adaptations seem to act mainly on the long bone of the big mammals, while in the case of small mammals the selective pressure seems to be exerted mainly on the metapodial bones (Fig. 3). On the other hand, it seems that the proportionality is more constant between the forelimb long bones than between the hindlimb ones, and the relative lengths of humerus and femur are more similar than those of ulna and tibia. If the whole legs are compared,

there appears a slight tendency to have a humerus + ulna longer than femur + tibia. In comparison to the data on the only big order of terrestrial mammals not studied here (Primates), the main disagreement with the results of AIELLO (1981) concerns the slope for the correlation between the lengths of both legs. Primates show a slightly negative allometry (0.95), while in the other orders of terrestrial mammals studied (see table 6) the relationship is isometric or positively allometric, except for *Canis* sp. About the relationship between diameters, only in two cases, the forelimb of dogs and the hindlimb of rodents, the diameters of the distal bones seem to scale faster than those of the proximal bones. Supposing that diameters are proportional to the strength of bones (see above), it appears that proximal and distal bones are submitted to more or less equivalent stresses. Something similar was assumed by means of experimental work on bending and twisting strength in small mammals and birds (BOU *et al.*, accepted).

According to the terminology and distinction introduced by JENKINS (1971), in the sample used in this study there are cursorial and non-cursorial mammals. However, it seems that no important difference exists from the point of view of limb proportions between those two types of quadrupeds, except the already commented characteristic of more variability within the metapodials than in long bones in small mammals, although no direct relationship appears.

Another question concerns the way in which we recognized the homologous structures in both limbs. As frequently happens in morphology, two possibilities exist : the structural and the functional. From a structuralistic approach, our comparisons are correct. But we cannot forget the different role of the two girdles in mammals : the main element of the pectoral girdle, the scapula, is capable of forwards and backwards oscillations. This fact and the fact that in some mammals (for instance, the cat) the motor pattern of the scapula long muscles and that of the muscles of the femoral region are identical, induce some morphologists to think of a functional « homology » between the scapula and the femur (Jean-Pierre Gasc, personal communication). If that point of view is right, a comparison between the lengths of the humerus and the tibia would seem reasonable. Let's do this comparison. According to our results for all the sample

$$\text{ulna} \; \alpha \; (\text{humerus})^{1.02}$$

and

$$\text{ulna} \; \alpha \; (\text{tibia})^{1.12}$$

Therefore

$$(\text{humerus})^{1.02} \; \alpha \; (\text{tibia})^{1.12}$$

or

$$\text{humerus} \; \alpha \; (\text{tibia})^{1.12/1.02} \; \alpha \; (\text{tibia})^{1.098}.$$

So, whether the comparison of the tibia is established with its structural (ulna) or functional (humerus) homologue, the result is a slightly positive allometry. That means that in all cases the forelimb bone scales faster than the hind limb bone.

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SHORT NOTES

POTAMOTHRIX VEJDOVSKY ET MRÁZEK, 1902 (OLIGOCHAETA, TUBIFICIDAE) : UN GENRE D'OLIGOCHÈTE DULÇAQUICOLE NOUVEAU POUR LA FAUNE BELGE

par

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RÉSUMÉ

Potamothrix bavaricus, P. hammoniensis et *P. moldaviensis,* trois espèces d'Oligochètes dulçaquicoles, ont été découverts pour la première fois en Belgique, malgré leur répartition holarctique. Cette découverte témoigne de la méconnaissance de ce groupe en Belgique. D'autres espèces de ce genre sont susceptibles d'être encore trouvées dans le futur. Des critères d'identification, basés sur les soies spermathécales sont proposés.

Mots-clés : Oligochètes dulçaquicoles, *Potamothrix,* identification

Potamothrix Vejdovsky and Mrázek, 1902 (Oligochaeta, Tubificidae) : a new genus of freshwater oligochaete for the belgian fauna

SUMMARY

Potamothrix bavaricus, P. hammoniensis et *P. moldaviensis,* three species of freshwater Oligochaetes, are recorded for the first time from Belgium, this in spite of their holartic distribution. The present discovery is an indication of the poor knowledge of this group in Belgium. Others species of this genus are likely to be found in the future. Identification criteria, based on the spermathecal setae, are proposed.

Key words : freshwater Oligochaetes, *Potamothrix,* identification

INTRODUCTION

Les Oligochètes ont toujours eu la réputation de groupe difficile (BRINKHURST, 1971 ; 1982) et peu attrayant en raison de leur apparente monotonie architecturale (GIANI, 1984). Bien que les difficultés soient essentiellement d'ordre technique et rendent l'observation des Oligochètes plus laborieuse que difficile (GIANI, 1984), il faut y voir l'origine de la méconnaissance de ce groupe en Belgique.

L'essentiel de la faune d'Oligochètes dulçaquicoles de Belgique est connu depuis le catalogue dressé par TÉTRY en 1940. Depuis lors, très peu de travaux y ont été consacrés et sur les 48 espèces actuellement connues, quatre seulement sont postérieures à ce catalogue (MARTENS, 1989).

Récemment, MARTENS (1989) a établi, sur base bibliographique, la première liste des Oligochètes d'eau douce de Belgique. Il ressort de cette étude que seule la faune aquatique particulière des grottes et des eaux saumâtres a été étudiée en détail, tandis que les eaux de surface n'ont été analysées que sommairement. Il est significatif, à cet égard, qu'il ait fallu attendre le travail de ANDERSON-DE HENAU (1980) pour découvrir en Belgique *Limnodrilus hoffmeisteri* CLAPARÈDE, 1862, et *L. claparedeianus* RATZEL, 1868, deux espèces cosmopolites, la première étant considérée comme le Tubificidae le plus commun et le plus largement répandu dans le monde (BRINKHURST et JAMIESON, 1971).

Sachant que BRINKHURST (1971) rapporte 83 espèces pour la faune britannique et que MOL (1984) en mentionne 77 pour la faune hollandaise (pour les cinq familles strictement aquatiques, soit Aeolosomatidae, Lumbriculidae, Haplotaxidae, Naididae et Tubificidae ; MARTENS, 1989), on peut en déduire qu'une étude plus approfondie des Oligochètes dulçaquicoles de Belgique révèlera encore de nombreuses espèces.

Le genre *Potamothrix* VEJDOVSKY et MRÁZEK, 1902, a été découvert lors d'un échantillonnage au filet troubleau dans la vase rivulaire de l'étang du Parc Léopold à Bruxelles en décembre 1990. Bien que vidé de son eau, l'étang contenait encore quelques poches d'eau stagnante recouvrant un sédiment noir fortement réduit, dans lequel deux espèces d'Oligochètes ont été trouvées : *Potamothrix bavaricus* (ÖSCHMANN, 1913) et *Limnodrilus claparedeianus*. Au cours d'une étude de la faune aquatique de la réserve naturelle du Blankaart (Flandre occidentale) en 1989, deux autres espèces de *Potamothrix* ont également été identifiées (MARTIN et VERDONSCHOT, 1991) : *P. hammoniensis* (MICHAELSEN, 1901) et *P. moldaviensis* (VEJDOVSKY et MRÁZEK, 1902). Ces espèces ont été prélevées au moyen d'un carottier (4,4 cm, superficie 15,2 cm^2), à quelques mètres du rivage sud-ouest de l'étang (22/6/89).

Ceci porte à trois le nombre d'espèces nouvelles pour la faune d'Oligochètes dulçaquicoles de Belgique et à 51 le nombre total d'espèces d'eau douce. Ces trois nouvelles espèces sont conservées à l'Institut des Sciences Naturelles de Belgique (No I.G. 27 759).

A nouveau, la découverte de ces trois espèces de *Potamothrix* témoigne de la méconnaissance des Oligochètes d'eau douce de Belgique puisque, parmi les dix

espèces formant la liste courante de ce genre (BRINKHURST et WETZEL, 1984), elles sont les seules à avoir une distribution holarctique (BRINKHURST et JAMIESON, 1971), bien que *P. bavaricus* soit considérée comme une espèce rare (BRINKHURST, 1971). Ce sont les seules espèces de ce genre connues en Grande-Bretagne (BRINKHURST, 1971), en Hollande (MOL, 1984 ; VERDONSCHOT *et al.*, 1982 ; VERDONSCHOT, 1981) et en Allemagne (UDE, 1929). Le genre *Potamothrix* est mieux représenté en France et en Suisse puisque, outre *P. hammoniensis* et *P. moldaviensis,* trois autres espèces ont été rencontrées, soit *P. heuscheri* (BRETSCHER, 1900), *P. bedoti* (PIGUET, 1913) et *P. vejdovskyi* (HRABE, 1941) (France : GIANI, 1979 ; JUGET, 1967 ; Suisse : LANG, 1989 ; JUGET, 1967). Etant donné que la répartition de *P. vejdovskyi* semble assez restreinte (lac Léman, lac du Bourget, Danube et Moldavie ; BRINKHURST et JAMIESON, 1971 ; JUGET, 1967), on peut raisonnablement espérer, à partir de ces informations, qu'au moins *P. heuscheri* et *P. bedoti* pourront être découverts dans le futur.

CARACTÈRES D'IDENTIFICATION

Les principaux critères d'identification du genre *Potamothrix* reposent sur les caractères anatomiques internes des genitalia (BRINKHURST et JAMIESON, 1971) dont la difficulté d'observation peut rebuter toute personne non spécialisée dans l'identification des Oligochètes. En pratique, comme l'aspect des soies spermathécales est caractéristique du genre *Potamothrix* (POP, 1971), leur forme et leur taille fournissent des critères suffisants pour l'identification des espèces signalées dans ce travail. Etant donné que les informations et illustrations concernant ces soies ne sont pas toujours très claires dans la littérature, nous en donnons ici une description complémentaire. POP (1976), BRINKHURST (1971) et UDE (1929) donnent des schémas des soies spermathécales de quelques espèces de *Potamothrix* qui compléteront utilement cette description.

P. moldaviensis est le plus aisé à reconnaître car, d'une part, il est le seul dans ce groupe à ne pas posséder de soies capillaires dorsales et, d'autre part, lors de la maturité sexuelle, les pénis font saillies au segment XI donnant à l'animal un aspect bien caractéristique (Fig. 1 ; LAFONT, 1983). Bien que ce critère suffise à lui seul pour identifier l'espèce, une soie spermathécale est représentée à la figure 2A à des fins de comparaison.

P. hammoniensis et *P. bavaricus,* deux espèces avec soies dorsales capillaires et soies dorsales pectinées, se différencient, quant à elles, sur base de leurs soies spermathécales (Fig. 2B et 2C). Dans le premier cas, la partie distale de la soie a des bords externes plus ou moins parallèles et, si sa longueur jusqu'à l'extrémité de la gouttière interne est pratiquement la même que chez *P. bavaricus* (environ 70 µm), sa hampe basale est beaucoup plus longue, donnant une longueur totale de 160 µm à la soie. Dans le second cas, la partie distale de la soie est plus large à sa base qu'à son extrémité, lui donnant une forme triangulaire, et la hampe est plus petite (longueur totale de la soie : 128 µm). Chez *P. hammoniensis,* la soie spermathécale

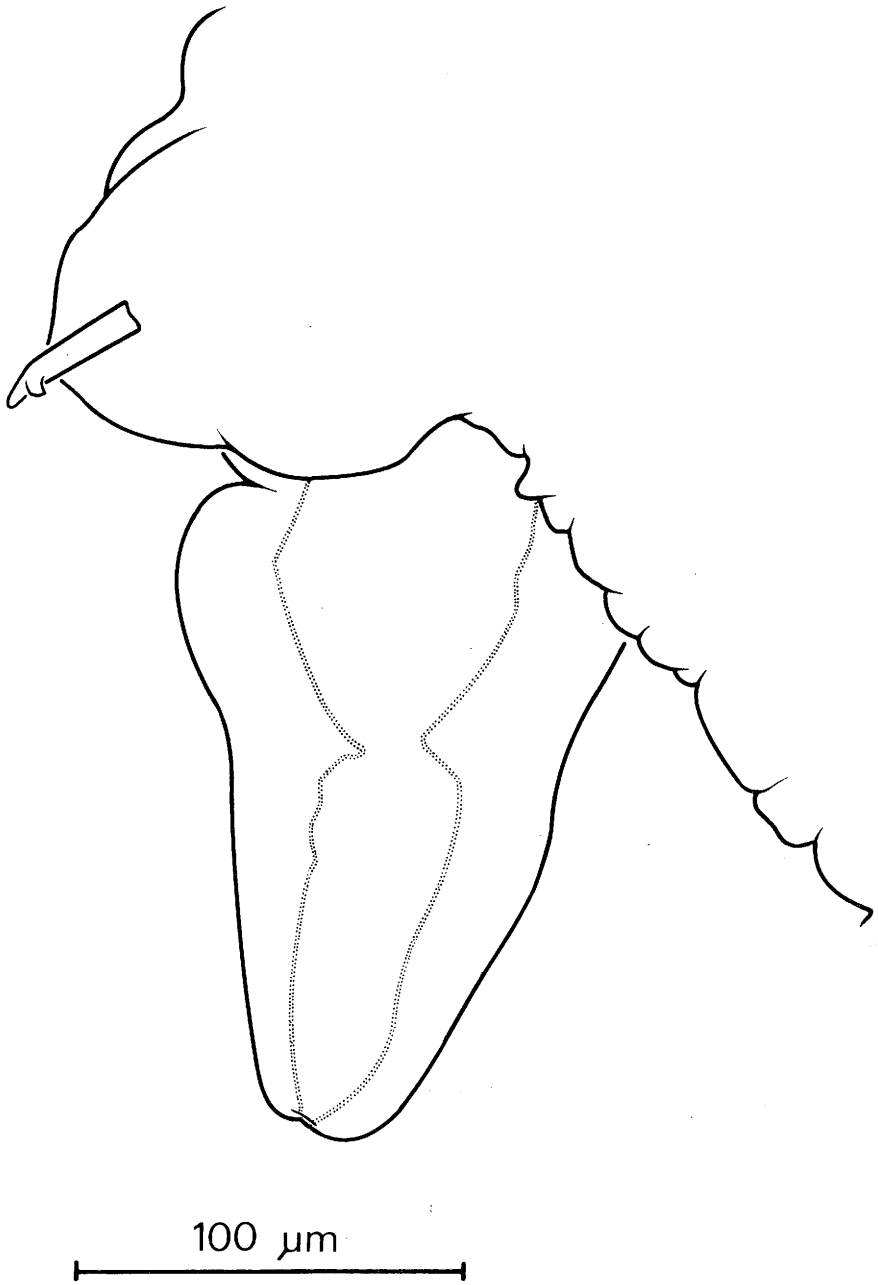

Fig. 1. — Saillie d'un pénis de *Potamothrix moldaviensis*.

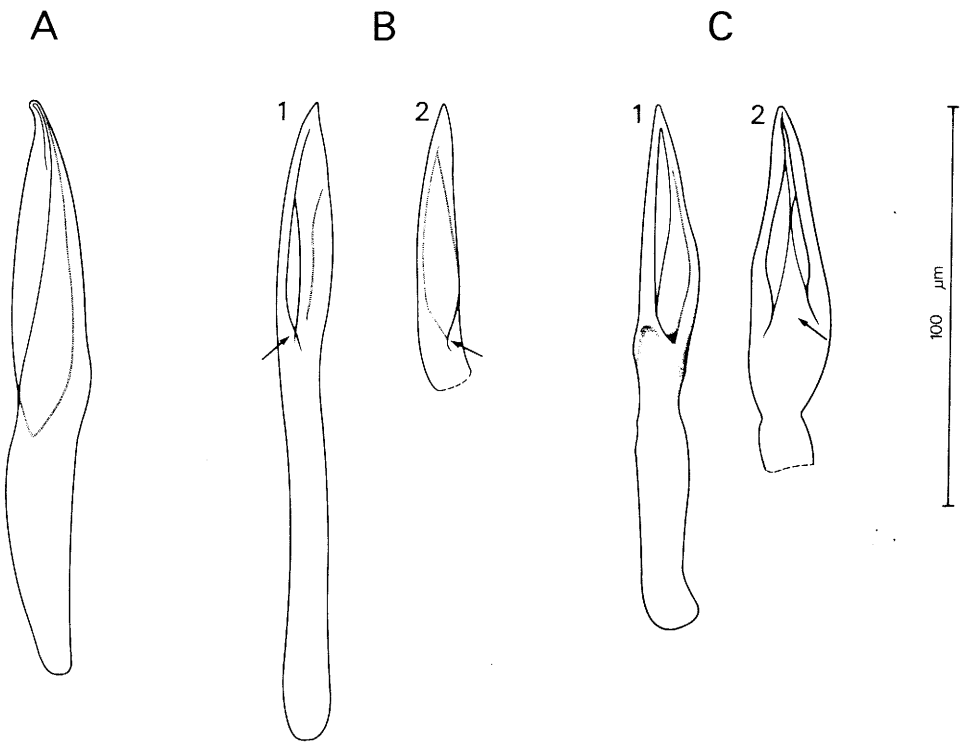

Fig. 2. — Soies spermathécales de *Potamothrix moldaviensis* (A), *P. hammoniensis* (B) et *P. bavaricus* (C), en vue trois-quart face (1) et frontale (2). Toutes les soies représentées appartiennent à des individus différents, y comprises celles représentées sous des angles de vue différents.

s'enroule sur sa partie distale, ainsi que chez *P. moldaviensis*. Par contre, chez *P. bavaricus,* elle reste ouverte sur toute sa longueur de sorte que les bords de la gouttière interne ne se rejoignent pas à la base, contrairement à la gouttière de *P. hammoniensis* et *P. moldaviensis* qui sont fermées aux deux extrémités (voir les flèches sur la figure 2B, C).

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