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PROGRESS REPORT 1975

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NETHERLANDS CENTRAL INSTITUTE FOR BRAIN RESEARCH - AMSTERDAM

Progress Report 1975

History of the Institute

In 1905, the Royal Netherlands Academy of Arts and Sciences applied to the Dutch Government for permission to found an institute for brain research. The government consented and on June 8th, 1909 the Netherlands Central Institute for Brain Research was seated in a wing of the then newly erected Department of Anatomy and Embryology of the Municipal University of Amsterdam. The institute was built under the direction of L. Bolk, professor of anatomy and embryology, who, together with the neurologist professor C. Winkler, was one of the most important supporters of the institute. The first director was Dr. C. U. Ariëns Kappers who became world-famous as a comparative neuroanatomist. After some years he was appointed professor of neuro-anatomy at the University of Amsterdam. After his death, in 1946, Kappers was succeeded by professor B. Brouwer, who previously had held the chair of neurology at the University of Amsterdam. Brouwer was primarily interested in neuropathology. During his management Dr. J. Drooglever Fortuyn, later on professor of neurology at the University of Groningen, introduced electrophysiology at the institute.

After the untimely death of Brouwer, in 1949, the Dutch government agreed that the institute should be reorganized and extended. Thus it became possible to found some new divisions enabling the institute to perform multidisciplinary research in the broad field of neurosciences, which was in accordance with its original aim.

In 1952, professor S. T. Bok, who previously had held the chair of histology at the University of Leiden, was appointed director. His merit has been further multidisplinary research at the institute on a large scale. He was one of the first researchers in the quantitative analysis of the brain, especially of the cerebral cortex, who earned great fame. After his retirement he was succeeded, in 1962, by J. Ariëns Kappers, previous professor of anatomy at the University of Groningen, who at the same time was appointed professor of neuroanatomy at the University of Amsterdam.

In 1964, the institute moved to a provisional, but much larger building and some additional barracks have since been built.

The institute is a governmental institution, with a total number of 72 staff places from which 35 for research workers. Its financial support is supplied by the government. It is managed by a director under the supervision of a board of professors of various disciplines at Dutch universities. Members of the board are appointed by the Royal Netherlands Academy of Sciences and Letters.

Recent developments

When Prof. Kappers decided to retire, Dr. H. G. J. M. Kuijpers, professor of anatomy at the Erasmus University in Rotterdam, was nominated by the Academy to succeed him as director of the institute. On the retirement date (August 1st, 1975) the succession had not been effectuated, however, since discussions with the government were still in progress regarding a new building, guarantees for the budget and

research facilities for Prof. Kuijpers research group. At the request of the government, Prof. Kappers remained on as temporary director. On August 15th, 1975, the members of the advisory board were informed unexpectedly that the government had decided to close down the institute as an economy measure. This decision (taken without having consulted any scientific body) was announced to the members of the institute on August 27th. A stream of national and international protests against this measure along with wide support for the efforts of the institute staff persuaded the Dutch parliament to request the government to reconsider the decision.

In the meantime Dr. D. F. Swaab, member of the staff of the institute, had been appointed as acting director. An independent committee - consisting of Prof. Dr. H. B. G. Casimir (chairman), Prof. Dr. D. de Wied and Prof. Dr. J. Joosse - is currently investigating the possibilities for continuation of the research work of the institute.

Research

The institute's research had been concentrated until recently mainly within the (discipline orientated) *departments*, e.g., neurophysiology, neurohistology and neurochemistry. The members of the various departments are listed below. This "horizontal" distribution according to techniques still has value, of course, especially where extensive common use of expensive equipment is involved. In addition, such departments gain importance because of the increasing demand of technical "service" to members of other departments. A drawback of this "horizontal" organization, however, has been all along the tendency for a large number of fragmented problems, that were often only very indirectly related, to be studied in a rather isolated fashion.

In the last few years, therefore, an alternative "vertical" way of collaboration has gained greatly in importance. This type of organization is centered around a relatively small number of research *projects*, in which investigators from different disciplines (biologists, physicists, biochemists, M. D.'s etc.) as well as from different departments are working together. For several projects this multidisciplinary collaboration has developed to a point where it is no longer desirable to present a progress report organized on the basis of the departments.

The scientific staff is striving to further stimulate this new approach in the direction of a *central theme* for the institute as a whole, which would serve to unify the diverse projects, or sub-themes. For the organization of conferences, work visits to foreign laboratories, lectures, foreign visitors, etc., the reader is referred to the year book (1975) of the Royal Netherlands Academy of Arts and Sciences.

D. F. Swaab

DEPARTMENTS

Director: Prof. Dr. J. Ariëns Kappers (until 1.8.'75 and temporary until 1.11.'75)
Dr. D. F. Swaab (temporary from 1.11.'75)

Department of Experimental Neurology

Head: Drs. P. A. de Groot
Dr. J. C. de Valois (until Dec. '75)
Drs. C. V. de Blécourt
Dr. J. P. Muizelaar (military serv.)
Dr. J. P. Schadé (until 1.1.'75)
Dr. W. van Emde Boas (until 1.1.'75)
Drs. J. H. de Groen (until 1.4.'75)

Department of Comparative Neurophysiology

Head: Dr. M. A. Corner
Dr. R. E. Baker
Drs. H. A. A. de Jong

Drs. W. L. Bakhuis
Drs. K. Boer
Drs. J. S. du Pont
J. C. M. v. d. Nes (stud. ass.)
H. Bour (stud. ass.)
J. W. L. Nolten
R. M. Nooy
A. Ph. J. Richter
J. Sels (secr.)

Computer Department
Head: Ir. J. Smith
Ir. K. Kuypers
P. Kolber (HTS-stag.)
E. Smienk (HTS-stag.)

Department of Behavior Physiology
Head: Dr. N. E. van de Poll
Drs. H. van Dis
J. G. van Oyen (stud. ass.)
B. Kouwenhoven (stud. ass.)
M. M. Smidt (secr.)

Department of Ethophysiology
Head: Drs. A. P. v. d. Mechè
Drs. J. P. C. de Bruin
Drs. B. Bermond
V. Nolten

Department of Histochemistry-Microchemistry
Head: Dr. D. F. Swaab
Drs. G. J. Boer
Drs. J. Dogterom
C. W. Pool (stud. ass.)
C. M. F. van Rheenen-Verberg
B. Fisser
J. v. d. Velden (secr.)

Department of Comp. and Quantitative Neuroanatomy
Head: Drs. H. B. Uylings
Dr. A. Miodonski
Drs. K. C. Hodde
Drs. P. Kenemans
Drs. W. A. M. Veltman
B. M. Przybylski-Zweesaardt
M. A. P. Vrielink-Van Dam
S. W. Lust-Bosboom (secr.)

Department of Electron Microscopy
Head: Dr. H. J. Romijn
Drs. F. W. van Leeuwen
Dr. J. Varela (until 1.1.'75)
M. Mud
P. Wolters

Department of Histology and Cytology
Head: Prof. Dr. J. Ariëns Kappers (until 1.11.'75)
Dr. A. R. Smith (until 1.10.'75)
Drs. P. Pevet
Drs. H. van Bronswijk (until 1.8.'75)
R. van Oosterom (until 16.10.'75)
S. L. Liem (until 1.10.'75, subsequently Dept. of Experimental Neurology)
P. Röring (secr.)

Department of Neurochemistry
Head: Dr. A. B. Oestreicher
Drs. C. van Leeuwen
J. Maas
M. F. J. Bosman

Department of Neuropharmacology
Head: Drs. M. van Wijk
Drs. L. van de Kar (until 1.8.'75)
I. Oberink (until 17.11.'75)
A. M. Lampe-Kraakman (until 1.3.'75)

Electronical workshop
J. Overdijk

Mechanical workshop
A. W. Kamstra
E. W. Moes

Administration
J. H. Oudshoorn
H. Sijtsma
M. Holzmann-Jacobs
P. A. M. v. d. Poel

Drawing Department
S. Berkelaar

Photography Department
Head: A. T. Potjer
B. Zijlma
T. C. Potjer

General technical service
Head: J. C. de Jong
L. Tibbertsma
C. de Groot

Household service
C. Salgado
M. A. Scheermeyer
J. N. Pals
H. H. Barbé
M. de Vos
C. de Haas

Animal care
Head: F. Harkema
R. Hofer

Library
C. Winkler

PROJECTS (and project leaders)

CYTOPHYSIOLOGY OF THE EPIPHYSEO-HYPOTHALAMO-HYPOPHYSEO-GONADAL SYSTEM

Participants

Prof. Dr. J. Ariëns Kappers (M.D., 10% until 1.11.1975)
Dr. A. R. Smith (M.D., 100% until 1.10.1975)
Drs. P. Pevet (biologist, 100% – from 1.10.1975 on in the department of electron microscopy of the Institute)
Drs. H. van Bronswijk (M.D., 100% until 1.8.1975)
Drs. L. van de Kar (biochemist, p.m. until 1.8.1975)

S. L. Liem (analyst, 100% until 1.10.1975)
R. van Oosterom (analyst, 100% until 16.10.1975)
I. Oberink (analyst, p.m. until 17.11.1975)

The work performed was related to a multidisciplinary investigation on the (cyto)physiology and endocrinology of the epiphyseo-hypothalamo-hypophyseo-gonadal system. Fluorescence histochemical, electron microscopical and biochemical changes in cells of organs constituting this system, more especially in pinealocytes and neurones of some hypothalamic nuclei, next to changes in serum hormonal levels have been investigated under various experimental conditions.

Theme 1: Experimental study of functional interconnections between the organs constituting the epiphyseo-hypothalamo-hypophyseo-gonadal system in the rat

On the ground of previous results obtained by part of the team and of literature data it appears that the pineal gland, or epiphysis cerebri, exerts its well-known antigonadotropic influence at least in part via the arcuate and ventromedial hypothalamic nuclei which belong to the so-called "hypophyseotropic area". However, very little is known about the feed-back mechanisms regulating the function of the pineal gland. By earlier investigations it had been shown that some pinealocytes and some neurones of the hypothalamic nuclei mentioned contain yellow fluorescent serotonin while others contain a peptide which is rich in tryptophan and is yellow autofluorescent. Administration of pCPA and castration had been demonstrated to change the numbers of yellow fluorescent and of yellow autofluorescent cells in the pineal gland and in the hypothalamic nuclei mentioned, respectively. In the arcuate and ventromedial hypothalamic nuclei, the numbers of these two cell types had also been shown to respond to pinealectomy and to pinealectomy followed by substitution using pineal extracts. In general, these earlier investigations indicated that the number of fluorescent serotonin containing cells and that of autofluorescent cells, present in the pineal gland and in the hypothalamic nuclei mentioned, can be used as parameters in studying the functional activity of the epiphyseo-hypothalamo-hypophyseo-gonadal system. The hypothesis was put forward that the serotonin containing neurones in the arcuate and ventromedial nuclei, increased by pineal functional activity, would inhibit, in these same nuclei, the production of LHRH.

In 1975 the investigation was continued by performing the following *experiments:*

a. Testing the influence of gonadotropic hormone administration, such as PMS, HCG, LH and FSH, and of castration on the absolute and relative numbers of serotonin containing fluorescent cells and of autofluorescent cells in the pineal gland using a special fluorescence technique.

b. Testing the effect of implantation, in the hypothalamic area concerned, of 5,6-dHT, 6-DOPA and of pineal tissue on the numbers of fluorescent and autofluorescent cells present in the arcuate and ventromedial nuclei and in the pineal gland using the same fluorescence technique. In addition, the effect of these implantations on the serotonin content of both hypothalamic nuclei was studied using an enzymatic isotopic microassay for serotonin on the one hand, and on the serum gonadotropic levels by means of a radioimmunoassay for LH and FSH on the other. The effect of pinealectomy and of pinealectomy followed by implantation of pineal tissue in the hypothalamus regarding the numbers of fluorescent and autofluorescent cells in the arcuate nucleus and the serum LH and FSH was also investigated.

c. Determination of the serum LH level short after pinealectomy using the radioimmunoassay method.

Results

a. The increase of serum gonadotropin levels due to either castration or administration of gonadotropic hormones in normal male rats did not effect the number of serotonin containing pinealocytes after periods of 4 and 7 days and after 3 weeks. The amount of pineal autofluorescent cells was, on the contrary, significantly increased.

b. After implantation of 5,6-dHT and pCPA, a decrease in number of serotonin containing neurones was observed in the arcuate and the ventromedial nuclei, while a significant increase occurred in the number of autofluorescent neurones. In the pineal gland, however, the number of serotonin containing pinealocytes was significantly decreased after implantation of pCPA in the hypothalamus only. Serum LH levels significantly increased after 5,6-dHT implantation, but not after implantation of pCPA. Serum FSH levels did not change significantly as compared to those of control animals after implantation of either of the drugs.

After hypothalamic implantation of 6-DOPA, a decrease in number of autofluorescent cells was observed in the arcuate and ventromedial nuclei as well as in the pineal gland while the number of serotonin containing cells seemed to be unaffected. Serum LH levels were not significantly influenced by 6-DOPA implantation whereas FSH levels showed a significant decrease.

After pinealectomy followed by implantation of pineal tissue in the arcuate nucleus, the numbers of fluorescent and autofluorescent neurones, present in this nucleus, returned to normal. After pinealectomy, the serum LH level was significantly decreased while the serum FSH level was not influenced by this operation. After substitution by pineal extracts, no restoration to the control value was observed.

c. As a preliminary result a decrease in the serum level of LH could be demonstrated at day 7 after pinealectomy.

Conclusions

From the above results it can be tentatively concluded that the alterations brought about by implantation of drugs in the arcuate hypothalamic nucleus exerts an influence on the content of fluorescent serotonin containing cells and of autofluorescent cells in the pineal gland which may indicate the presence of a feed-back mechanism. In addition, the experiments performed, although supplying evidence that serotonin inhibits LHRH production, point to a somewhat more complicated functional mechanism that was hypothetized before. Dopamine may be specially involved in the hypothalamic regulation of FSH production in the hypophysis.

Investigations on this theme have now been stopped at the Institute because Prof. Dr. J. Ariëns Kappers, Dr. A. Smith, H. van Bronswijk and L. van de Kar left. The latter will continue research on this subject in the United States.

Theme 2: Investigation on correlations between the ultrastructure of Pinealocytes and the periods of the sexual cycle in mammals living in natural conditions, the mole and the mole-rat

So far, only few authors examined the possible correlation between pineal morphology and the functional state of the hypophyseo-gonadal axis which was the subject of the present theme.

The ultrastructure of pinealocytes has been studied at different periods of the annual sexual cycle of some mammals living in natural conditions. Special attention was given to those cell organelles involved in secretory activity, such as the Golgi apparatus, the endoplasmic reticulum and secretory vesicles. As it is known that the quantity of environmental light is a factor determining the functional state of the pineal gland and that photic stimuli are mediated by the lateral eyes, central nerve fibres and the sympathetic innervation to the pineal organ, pinealocytes have been studied in animals in which the eyes are not functional, i.e., the mole and the mole-rat. For this investigation, conventional electron microscopic techniques have been used.

Results

The pinealocytes, especially those in the mole, are characterized by very distinct changes in the shape of the cell organelles according with the periods of the sexual cycle. This is specially evident regarding the ribosomes, polysomes and the granular endoplasmic reticulum. Mole and mole-rat pinealocytes appeared to be much more active than those of mammals studied so far, such as rat and rabbit.

A number of pinealocytes of these blind animals are characterized by the presence of cysterns in the granular endoplasmic reticulum containing accumulations of proteinaceous material which have never been described in any other mammalian pineal organ. Possibly, the presence of this material is due to the animals life in complete darkness and may point to an exacerbation of some synthetic process, also existent in other mammals as is suggested by earlier results and those obtained in theme 1.

Conclusions

Clear evidence of a correlation between ultrastructural features of cell organelles, specially those involved in synthetic secretory activity, in pinealocytes and the periods of the annual sexual cycle has been obtained. Accumulations of proteinaceous material present in cysterns of the endoplasmic granular reticulum have been described for the first time. So far, the exact nature of this material has not yet been demonstrated.

PLASTICITY IN CNS

Participants
Drs. H. B. M. Uylings (biologist 100%)
Drs. W. A. M. Veltman (physicist 50%)
Dr. A. Miodonski (neurohistologist, physician 25%)
B. M. Przybylski-Zweesaardt (analyst 30%)
S. W. Lust-Bosboom (secretary 15%)
M. A. P. Vrielink-van Dam (technician, 20%)

Theme: Quantitative description of nerve cell branching patters in the cortex of the rat and of their development under influence of different life conditions

The integrative capabilities of neurons are in some way related to dendritic branching patterns. The functional morphological study of these patterns is therefore important. During the development of new quantitative analytical methods for describing the branching pattern of cortical dendrites, indications were found for the hypothesis that dendrites of *adult* mammals are still capable to form new branches, which is in contrast with the present opinions. These indications appeared, therefore, to be so valuable that further research was directed in this particular aspect. Questions for the present research are given in the titles of the two themes.

Theme 1: Are dendrites in the cortex of adult rat capable to form new branches in response to the changes in living conditions specially to a more stimulus-rich environment

This investigation is performed in collaboration with Prof. M. C. Diamond (University of California, Berkeley, USA). This year, the dendritic measurements are finished by Uylings during a 6-month visit to the laboratory of Prof. P. D. Coleman (University of Rochester, N.Y., USA). These measurements are punched into paper tapes in a certain format.

The analysis of the paper tapes is in progress with the aid of the computer present at our Institute. The analysis will be finished by Veltman and Uylings in the beginning of 1976.

Theme 2: Is recovery in the adult stage possible after retardation in outgrowth of nerve cell processes due to stunting environmental factors during the so-called vulnerable period in postnatal growth by putting the animals in an adequate stimulating environment?

Stunting environmental factors are, *e.g.*, environments poor in variation and possibilities of contacts, disturbances in thyroid gland function and undernutrition. Furthermore, the retardation in the outgrowth of nerve cell processes could possibly be associated with insufficient cortical blood supply. For this, the blood vascular pattern (angioarchitecture) can be indicative.

Before starting research to answer this question it has been appeared necessary to evaluate the preparations obtained by different Golgi methods. The crucial point here is: which Golgi method(s) stain(s) the branching patterns of nerve cell processes during development completely and aselectively. We will start, therefore, a developmental study of the rat brain for comparing the Golgi-Cox and the Golgi-Kopsch method (especially with Dr. Miodonski) at the end of 1975. At the same time Dr. J. G. Parnavelas (University College London, England) will start a developmental study comparing "rapid Golgi" with the E.M. method and probably also with a combined Golgi-E.M. technique. This allows a comparison of the various staining methods.

In view of the possible association of the blood supply and the outgrowth of nerve cell processes, a pilot-study will start (by Miodonski) to combine, in the same tissue, the staining of the cortical blood vascular pattern and the Golgi staining of the nerve cell processes (see also his project).

The results of the above-mentioned developmental studies will be combined with the results of the developmental electrophysiological study of Dr. M. A. Corner.

For the quantitative elaboration of the comparison of the above-mentioned Golgi methods as well as for the study of the development of the branching pattern of dendrites (and that of cortical blood vessels) many measurements are necessary. Therefore, we, J. Overdijk (electronic workshop), A. Kamstra (mechanical workshop), and the computer section are constructing a semi-automated system for measuring branching patterns. In this, our experience with the semi-automated system of Prof. Coleman was most valuable. The constructing phase will be finished in the course of 1976. When this system is ready, the time necessary for doing measurements will be shortened for about $\pm$ 20 times, so that much more investigations per time-unit are possible.

DEVELOPMENT OF THE CNS AND BEHAVIOR

Participants
Dr. M. A. Corner (biologist, 100%)
Dr. R. E. Baker (biologist, 100%)
Drs. W. L. Bakhuis (biologist, 100%)
Drs. K. C. Hodde (physician, 20%)
Dr. A. Miodonski (physician, 15%)
Dr. H. Romijn (biologist, 10%)
Drs. W. A. M. Veltman (physicist, 50%)
H. Bour (student assistant, 50%)
J. van de Nes (student assistant, 50%)
R. Nooy (technician, 30%)
A. P. J. Richter (technician, 100%)
J. Sels (Secretary, 65%)

(Falls in part under "FUNGO" workgroup no. 13-24-33: "Growth and Development", section *Brain and Behavior*).

A number of themes are presented here, all dealing with one or another aspect of nervous system ontogenesis. In addition three key ideas are implicitly discernible running throughout the project as a whole, albeit with different emphasis from one theme to the other. These ideas are:

a. *basic neuronal circuitry* underlying stereotyped patterns of functional activity in developing neural networks, whether this be brainstem phasic discharges (fetal motility, "REM"-like activity during sleep: (I) themes 1–3), cortical slow potentials (spike-and-wave, "trace alternant": (II) themes 4–5), or spinal cord reflex responses ((III) themes 6–8);

b. *endogeneous neural excitation*, often highly rhythmical, generated by developing neurons and which either expresses itself directly as "spontaneous" behavior and/or brain waves, or else modulates the responsiveness to sensory stimulation of central neuronal networks;

c. *neuronal growth processes* ("plasticity") as regulated by their own functional activity, both endogeneously generated and sensory-evoked, and the importance of this for the maturation and maintenance of the organization of the nervous system.

Theme 1: The neural regulation of avian hatching behavior

As a contribution to the understanding of the interrelation between behavioral and neural development, the study on the last part of embryonic life of the chicken (hatching) has been continued.

A system for automatic registration and data processing has been developed which enables registration of some aspects (intervals between —and duration of— motility bursts) of the hatching behavior for many hours at a stretch, the animal being artificially prevented from escaping its eggshell. The influence of temperature on hatching behavior has been studied with this system, and it was found that an ambient temperature

of 33 to 34° C is optimal for regular and prolonged manifestation of the movement bursts. This is 4° C below normal (incubation) ambient temperature.

The modal values both of burst-to-burst interval distributions and of the burst duration distribution remain fairly constant over a 6-hour registration period, although the frequency of bursts per hour declines (from about 300 to 30) during that period. These are thus two parameters which well characterize the stereotypie of this "fixed action pattern". At the optimal temperature (33–34° C) the bursts continue for about the first 2 hours, without any interruption, at a frequency of 4–6 per minute. Therefore, this preparation is very useful for studying (even slight) effects of experimental manipulations.

The following studies are being done, or will shortly be started, with this preparation to evaluate the effects on hatching behavior of:

1. different body temperatures: a follow-up of the above-mentioned ambient temperature study;

2. hormones that are suspected to play a role in hatching behavior: this study will be done in co-operation with the department of neuro-endocrinology where the role of the fetal brain during parturition has been studied;

3. drugs which are known to influence the states of vigilance in the neonate chick: to complete the vigilance experiments mentioned in the Progress Report of 1974, which are being done in collaboration with Prof. M. Jouvet (Department of Experimental Medicine, of the Claude-Bernard University of Lyon);

4. different degrees of freedom-of-movement of head and limbs, to study the possible role of propioceptive input: the results up till now indicate that a much reduced freedom is a condition for the stereotyped sequence of movements and for the duration of bursts, while the rhythmic aspect of hatching behavior is maintained even during greater degrees of freedom;

5. localized lesions, faradic simulation: to locate the brain areas responsible for hatching behavior; for this purpose recordings of the electrical activity of the relevant brain areas will be also made. This theme forms part of the thesis of W. L. Bakhuis.

Theme 2: Cyclic EEG and motility patterns during sleep in infant rats

A study was begun last year—based upon spontaneous behavior patterns observed in the chick *in ovo*, and during hatching and *sleep* in later life under certain conditions: see theme 1; and Corner, Bakhuis and Van Wingerden: in "Behavioral Embryology, p. 345, 1973—on the motility of rat pups restrained in a quasi-fetal posture. Such preparations often showed strikingly similar bursts of spontaneous stereotyped total-body movements as in the chick, clustered as a rule in distinct "activity phases"

at intervals of several minutes. The EEG data obtained last year were processed this year by the computer department, and revealed a highly negative correlation between EEG slow wave activity and the amount of burst motility. Since the animals appeared to be in a sleep-like state throughout, despite the vigor of their movements, the term "*R*apid *B*ody *M*ovements" was coined to describe this phenomenon. In a paper accepted this year for publication in the "EEG Journal" we have suggested that the RBMs are a physiological antecedent of the well-known REM discharges during sleep in the adult. They are themselves, in turn, probably a continuation of fetal motility patterns into post-natal life. Continuation of this problem is conceived via the application of nerve tissue culture as a "model" system for primitive behavior control networks (see theme 3).

Theme 3: Spontaneous swimming rhythms of frogs at larval stages

Motor burst activity has been observed not only *in ovo* (theme 1) and during sleep (theme 2), but also in many types of neuro-muscular preparations cultured *in vitro*. Tissue culture thus can provide a useful model system for studying the genesis of spontaneous behavior patterns (Corner and Crain: J. Neurobiol., *3*, 25, 1972). Stereotyped bursting is often modulated, furthermore, by oscillations lasting several minutes which result in a distinct rest-activity cycle. Such patterns of activity were thus postulated to represent basic "units of function", characteristically generated by primitive neural networks. In order to examine the significance of such activity patterns in the evolution of behavior (Corner: Develop. Psychobiol., in press), spontaneous swimming in larval anurans (amphibia) was investigated.

Three different species of frog gave similar results: spontaneous motility occurs in the form of brief bursts, clustering rhythmically at intervals of several minutes. The bursts are usually somewhat variable in duration, and in the intervals between them, but episodes of highly regular or stereotyped bursts are also quite often observed. During behaviorally quiescent epochs, which may be very prolonged, the animals sometimes even come to lie on their sides in the water as if "asleep". Our tissue culture model for embryonic motor patterns now offers a possibility for analyzing the neurological basis for these stereotyped and periodic discharges, by offering a suitably simplified system in an essentially constant milieu. In particular, the origin of ultradian ($\pm$ 1 hr) rhythms described as the "Basic Rest-Activity Cycle" during sleep in higher vertebrates, including man, could be tackled by the use of various combinations of neural structures *in vitro*. Finally, the potential *importance* of all this spontaneous bioelectric activity for the normal maturation and maintainance of functional organization in the nervous system is to be investigated under rigorously controlled conditions.

Theme 4: Early development of spontaneous electrocortical activity (EEG) in the albino rat

In a previous EEG study using the chick embryo (Corner, Roholl and Bot: Exper. Neurol., *44*, 22, 1974) we showed that the period of most rapid development—attaining an essentially mature slow-wave sleep pattern—is probably under control of cerebral glutamate metabolism. The chick EEG is characterized by a succession of stereotyped high-voltage biphasic waveforms appearing at 17 days of incubation. In order to examine the possible generality of these findings, a similar experimental paradigm was applied to the infant rat, which is known to also show a very sudden maturation of the SWS pattern in the cortical EEG, occurring at about 10 days postnatally. Our results with glutamate injection prior to the 10th day pointed towards the same conclusion as for the chick embryo: glutamate triggered a premature appearance of high amplitude EEG waves, preferentially in the delta frequency band. This voltage increase failed to alter the characteristic rhythmicity ("tracé alternant"), however, by which the early rat EEG proved to differ convincingly from that of the chick. The tracé rhythm was highly regular, with a cycle of 1–2 min being evident in the time plots; the stored amplitude measurements are soon to be further processed by the computer department in order to better quantify this rhythm (autocorrelogram technique) and to search for it at later stages of maturity, when to the eye the EEG appears to have become essentially stationary.

(The continuation of this project would be desirable in conjunction with the NCIH project "Neuroplasticity" (Uylings/Veltman): see project 5 for details).

Theme 5: Synaptogenesis in the embryonic chick cerebrum

Our earlier multi-disciplinary study of brain development in the chick (Corner, Smith and Romijn: in "Ontogenesis of the Brain", vol. 2, 21, 1974) showed that cerebral dendritic and metabolic development did not closely parallel functional maturation as reflected in the spontaneous EEG. An electronmicroscopic study was therefore undertaken, completed this year in collaboration with Dr. Romijn, to see if the development of synaptic structure might not give some clues to the electrophysiological stages.

This would be especially interesting in view of the possibility that glutamic acid, implicated in earlier findings (see theme 4) was mediating its effect in ontogeny via synaptic transmission. The ultramicrographs gave no indication either that new synapses were being formed during the period of rapid EEG development, or that those already present underwent any significant further maturation at that point. On the other hand, a most dramatic explosion of *de novo* synaptic formation and enlargement occurred a few days later, after the slow-wave EEG pattern

had already become stabilized. This new development was correlated with the appearance of dendritic spines, and paralleled the first spontaneous *arousal* effects in forebrain electrical activity. The question is posed hereby of the *origin* of these new axonal endings in the chick cerebral "cortex", and of their presumed *role* in inducing the final differentiation of the dendrites with which they synapse. This problem could be profitably attacked using anatomical axon-tracing techniques, already in use in the NCIH neuroanatomy section, in combination with electronmicroscopy.

A further question which was formulated this year on the basis of the above results is the possibility that these late cyto-morphological events, surely of importance in the full development of cerebral function, are themselves a function of the organized neuronal activity (e.g., high amplitude slow-wave EEG pattern) which precedes them. The importance of spontaneous bioelectric discharges (as during REM-sleep) for normal neural development has long been suspected but is so far undemonstrated. Yet, this must be regarded as an important baseline parameter in considering the possible mechanisms by which *sensory-evoked* brain activity can exert its well-known ontogenetic effects. As such, this theme complements the project "Neuroplasticity", where dendritic development and the effects of sensory stimulation thereupon are being studied quantitatively. An electrophysiologist ought ideally to be added to such a group, both to monitor the physiological variables being manipulated and to study the functional correlates of observed morphological effects (*vide supra*). Finally, the nerve tissue culture model system (see project 3) offers a useful potential extension of this project, now that Golgi staining as well as E.M. studies are possible *in vitro*. This is because (1) better control can be achieved over the independent variable (i.e., level of functional activity) and also over crucial environmental parameters, and (2) the resulting effects upon neuronal activity, correlated with eventual structural changes, will be more precisely localizable.

Theme 6: Reflex ontogeny in skin-grafted frogs: role of cutaneous stimulation

The skin rotation preparation was worked out this year in a new species of anuran, *D. pictus*, which we are able to obtain the whole year round (courtesy of the Hubrecht Lab., Utrecht). Misdirected responses to cutaneous stimulation proved to arise in this animal from belly skin grafted on the back, but not vice versa. The possible importance of experience with cutaneous stimuli during the period of reflex stabilization was systematically investigated, and it was demonstrated that this was not a crucial factor: the reflexogenous skin area expanded and consolidated between 2 and 3 weeks post-metamorphosis in animals not subjected to any test stimulation whatsoever. Daily testing during 2–4 weeks post-met., on the other hand, resulted in a long delay in the maturation of the definitive (i.e., misdirected) behavior pattern. Prior to this time the

animals behaved as if the skin were *doubly*-innervated, and that the opposing potential responses were being elicited on a probabilistic basis. This presumably abnormal situation was suggested, in a paper submitted for publication in Develop. Psychobiology, to result from the presence of some nerve fibers in the skin already at the time of operation (Nafstad and Baker: Z. Zellf. *139*, 451, 1973). An experiment was designed to test this point, involving operations performed at embryonal stages—prior to the establishment of peripheral nerve connections—and will be started as soon as the next clutch of frog eggs becomes available. The application of our tissue culture preparation for amphibian material (Corner and Crain: Experientia *21*, 422, 1965) is also planned within this project, whereby the role both of nervous function and of peripheral innervation in the establishment of sensory connectivity patterns within the CNS can be analyzed under strictly controlled conditions.

Theme 7: Representation of the body surface in the dorsal root ganglion in frogs

A critical experiment was designed this year for testing the cutaneous modulation hypothesis, as opposed to selective (re)outgrowth as an explanation for the misdirected response phenomenon (see project 6; and Baker: in "Chemical Neurobiology", in press). This strategy is based upon the fact that, in the cat, cutaneous receptive fields are known to map somatotopically onto the dorsal root ganglion (the validity of the paradigm was then verified in consultations with several of the leaders in this field of research: R. Gaze and M. Keating, London; R. K. Hunt, Baltimore). The preparation has been worked out this year, using juvenile frogs, and it has proven possible to record unit responses to tactile stimuli over many hours. Each ganglion cell has a discrete receptive field, and each spot on the skin is multiply innervated by units of varying threshold and receptive area. All respond to stimulus movement rather than to steady touch, at a firing rate roughly proportional to the rate of stimulus displacement. There proved to be a wide overlap in the projections of belly and back skin units onto the ganglion in *R. esculenta*, and this finding is now to be checked in *Discoglossus*—and for each functional class of neurons separately. The nature of the changes of cutaneous projections in frogs showing misdirected reflexes (i.e., peripheral vs. central switching of afferent nerve connections) will then be able to be determined; the animals prepared this year for that purpose will soon be sufficiently large for electrophysiological recording studies.

Golgi impregnations of the ganglion cells were attempted with some success in the frog tadpole, and a developmental series is almost ready for examination. These preparations are to be used for morphological verification of the physiological projection patterns. (An attempt to do this by means of horseradish peroxidase uptake has so far been without success).

Theme 8: Central projections of sensory nerve fibers in the frog

Innervation patterns in the spinal cord are undoubtedly the ultimate key determined. Hitherto, such counts had been restricted to the central root or sensory ganglion. We have found that the ingrowing dorsal root fibers follow a similar developmental course as for the ganglion cell bodies and for the motor nerve fibers, viz., a steady increase in fiber number during the late larval stages, followed by a sharp decline immediately following metamorphosis. A pilot-study has also been completed for applying fiber-degeneration tracing techniques to the central projections of the afferent fibers. Single-skin type animals have been prepared (i.e., all-back or all-belly; Baker: Nature *236*, 235, 1972) in order to uncover any differential projections from these two skin fields, and also to localize the innervation fields for this sensory modality: it is not known for instance, if *individual* afferent fibers bifurcate to make both descending and ascending cord projections. (This is relevant to the possibility that the postulated central synaptic readjustment, under influence of the peripheral end-organs, involves a switching "on" or "off" of anatomically already present terminals). The silent-synapse hypothesis can be tested by comparing the electrophysiological with the morphological projections from cutaneous receptive fields.

A strategy was also worked out (and confirmed in consultation with the spinal cord physiologist, P. D. Wall, in London) for *directly* establishing the occurrence of an eventual "plastic" change in functional connectivity at the first synaptic relay station. This approach makes use of unit studies in single-skin type animals (*vide supra*), to locate abnormal projections of both belly and back receptive fields onto individual interneurons, correlated with the abnormal motor responses.

Students/guest workers

R. Scholte (student biochemistry): "Development of rhythmical electrocortical activity in the albino rat".

H. Bour (student biology): "Polygraphic investigation of sleep and wakefulness in the chick".

VISUAL SYSTEM OF THE FROG AND FROG SKIN RECEPTORS

Participants
Drs. J. S. du Pont (biologist, 100%)
R. Nooy (technician, 40%)
J. Sels (secretary, 15%)

Theme 1: Visual system of the frog

In the course of the present year the data of this theme did reach its final stage. The results were presented in four papers. Two of them are dealing with the optics of the frog's eye and furnished the following data:

1. data to compare a schematic eye in paralysed and non-paralysed conditions;

2. the computation of a schematic dioptric apparatus;

3. the consequences for the image forming on the retina with and without correction lenses in paralysed condition;

4. a possible manner of accomodation;

5. the estimation of the visual fields of one eye and of both eyes;

6. a comparison of under water vision to vision in air;

7. the width of the modulation transfer function of the frog's eye was estimated assuming that the point spread function is Gaussian. The width was estimated from measured intensities of the centres of images of objects (discs and bars). Those intensities can be described using easily computed expressions. The accuracy in width is better than 1%. The influence of agents like Flaxédil was found to be negligible.

The two other articles deal with the properties of metal microelectrodes and the relation between photically evoked potentials in the tectum of the frog. The results and conclusions can be summarized as follows:

1. The voltage-current relationship of the electrode is linear in the domain this electrode is used.

2. The frequency response curve and the phase characteristic of the electrode and amplifier is also linear.

3. The latency of the slow-potential is always shorter than that of action potentials in all stimulus situations.

4. There are stimulus situations and temperature conditions in which only the slow potential occurs.

5. The slow potential arises at the terminals of the optic nerve fibres in the tectum.

6. The action potentials originate at the myelinated optic fibre endings or the dendrites of the tectal cells.

7. If the dendrites happen to be the source of the action potentials then it is incorrect to ascribe functional characteristics of ganglion cells in the retina to sources of action potentials in the optic tectum and vice versa.

8. If the terminations of the myelinated fibres are the source of the action potentials then only 4% of the optic nerve fibres produce these action potentials.

Theme 2: The receptors in the skin of the frog

This theme is a part of the project "Development of the CNS and behavior". A possible explanation of the arising of the misdirected reflex might be that there exists a difference in the functional organization

between the receptors of the back skin and belly skin. These possible differences may concern the shape of the action potentials in the impulse patterning (trains of action potentials) and in the receptive field organizations such as the form of the field and the threshold distribution over the receptive field.

An isolated skin preparation is chosen so that it is possible to record simultaneously from back and belly skin fibres. For this preparation the mechanical workshop has developed a set-up that enabled us to record from all possible skin fibres and besides to regulate the temperature that is necessary for quantitative research. Moreover a precise receptive field plot is made possible with aid of this device.

For the threshold measurements a home-made hardware detection criterion (L. detector) was made operable and verified. A gross idea of the threshold distribution was obtained on the base of preliminary experiments.

NEURONAL FUNCTIONAL RELATIONS WITHIN THE EQUILIBRIUM SYSTEM OF PULMONATE SNAILS

Participants
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Dr. H. van Wilgenburg (neurophysiologist, advisor)
Drs. J. S. du Pont (biologist, advisor)
Dr. T. A. de Vlieger (neurophysiologist, advisor)
R. Nooy (technician, 20%)
J. Sels (secretary, 20%)

Signal transmission of statocyst information in the pulmonate snails Helix pomatia and Aplysia limacina

The present project is aimed at adding information about what is going on in the nervous system (CNS) to the existing studies which describe the behavior in terms of input/output relations of the animal as a whole. In my earlier experiments a set-up was realized enabling the registration of neural responses which were the results of minute differences in rotation speed and spatial orientation of the preparation. This recording chamber offered the possibility to make four simultaneous recordings from the right and left static nerve and from the right and left cerebro-pedal connective (cpc). This offered the advantage of being able to follow the statocyst activity at both the pre- and postganglionic level. This information can be used as the most important input for the spatial orientation of the animal and its compensatory movements.

Receiving an input from few sensory units, only 13 at each side, the animal can realize minute and precise compensatory movements and can maintain its balance. In order to give more distinct description of this signal transmission in the snail brain, the following scope of analysing techniques was practiced and further developed in 1975:

Frequency-time histograms of both pre- and postganglionic recordings (static nerves and cerebro-pedal connectives).

Amplitude-frequency histograms of static nerve activity, showing the average frequency of the various distinguishable sensory units.

Amplitude window discrimination resulting in a distinct and preconceived single statocyst unit from which the data, obtained during experimental conditions were analysed. The detection of units in the postganglionic nerve recordings is much more complicated, due to the presence of many more fibres.

Tilting of the preparation and recording the activity at two sites of the cpc resulted in an enhanced spike activity. Besides the predicted increase in pedal-directed spike activity there was also an unpredicted but noticeable increase in cerebral-directed spike activity.

The above mentioned individually detectable units from the statocyst induced a firing pattern of 1–5 consistent action potentials in the connective during the first 4 seconds of tilting. This result was obtained with the *average response* technique. After the recognition of these spikes in the analog recording, their amplitude and propagation velocity could be analysed. These two parameters that typify the individual action potential have been subjected to a combined *amplitude-window* and *time-window* procedure.

This detection technique has been developed in order to separate the units, directly involved with the preganglionic sensory spike potential, from those units which also show an increase in activity during tilting and those which present the total background activity. This preparation together with the analytic detection techniques as described above present a most suitable scientific substrate for further studies on basic neuronal problems dealing with sensory input and signal transmission.

NEUROENDOCRINOLOGY

Participants
Dr. D. F. Swaab (M.D., 100%)
Drs. G. J. Boer (biochem., 100%)
Drs. K. Boer (M.D., 100%)
Drs. J. Dogterom (biochem., 100%)
Drs. F. van Leeuwen (biologist, 100%)
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Drs. M. Visser (gynaecol. in service of the University of Amsterdam, 50%)
C. M. F. van Rheenen-Verberg (analyst, 100%)
B. Fisser (student-analyst, 100%)
J. W. L. Nolten (analyst, 50%)
R. M. Nooy (technician, 10%)
P. J. Wolters (technician, 35%)
J. v. d. Velden (secretary, 95%)

In the present project the interaction between brain and hormones is investigated. The two aspects of this project are: a) hormone production by nerve cells (neurosecretion) and b) the action of hormones on the brain. These aspects are essential in many processes: e.g., diuresis, growth, lactation, parturition, brain development and memory, and therefore of direct importance for the etiology, diagnosis, and treatment of diseases like diabetes insipidus, toxaemia of pregnancy, disturbances of parturition, lactation and growth and mental retardation. In this multidisciplinary project various departments of the Institute (dept. of histochemistry, comparative neurophysiology, electronmicroscopy and the computer department) are involved as well as clinics (i.e., dept. Obstetr. and Gynaecol., Univ. of Amsterdam) and other university laboratories.

This project is incorporated in the FUNGO projects nrs. 13-35-07, 13-35-04 and 13-35-10.

Theme 1: Immunolocalization of vasopressin and oxytocin

The exact sites of production and release are not established for any of the neurohormones. They are of importance because *e.g.*, the place of release might allow a particular function. For the action of vasopressin on memory for instance, a release of this hormone into the cerebrospinal fluid is supposed (v. Wimersma Greidanus *et al.*, 1975), as well as a release directly into the brain (Sterba, 1974). For the possible action of vasopressin as a releasing factor on the anterior lobe of the pituitary the release of vasopressin should take place into the portal vessels. In order to be able to solve this kind of problems we have developed very specific and sensitive immunolocalization techniques. These techniques are, in addition, essential for various themes mentioned below.

a. *Immunofluorescence technique.* By means of this technique already various hormones (vasopressin, oxytocin, αMSH, ACTH) were localized in the pituitary or the brain. In addition it was possible to quantify the potency and cross-reactivity of the many aliquots of antibodies raised against these hormones obtained by our group in the last few years. The development of a suitable model system consisting of synthetic hormones coupled to agarose beads, enabled us to show that immunolocalization techniques, used all over the world, do not sufficiently take into account the problem of cross-reaction. We have been able to solve this problem for the antibodies against the hormones mentioned above, by the development of a purification procedure, using the cross-reacting hormones on agarose beads. These procedures have shown to be of general use for the characterization and purification of many kinds of antibodies and are currently also applied in other laboratories. By means of purified antibodies, the exact localization of oxytocin and vasopressin in the hypothalamus has been established in normal Wistar rats and in rats

being either homo- or heterozygous for a hypothalamic form of diabetes insipidus (Brattleboro rats).

Within the hypothalamo-neurohypophyseal system the oxytocin and vasopressin containing cells were found in similar percentages in the supraoptic (SON) and paraventricular nucleus (PVN). Since the SON appeared to have 2.5 times more cells than the PVN, the SON contains most of the vasopressin *and* oxytocin-containing cells. This is against the classical view on the functional differentiation between the SON and PVN. Within the SON and PVN, oxytocin-containing cells were mainly localized in the frontal part and vasopressin cells more in the caudal part. Furthermore, vasopressin was found in the rat outside the hypothalamo-neurohypophyseal system in the suprachiasmatic nucleus. Whether this can be explained by storage of vasopressin, or actual synthesis of this hormone in the cells will be studied by immuno-electronmicroscopy. This technique will also be used for the precise immunolocalization of the vasopressin releasing sites (third ventricle, portal vessels or elsewhere in the brain).

For various reasons a comparison is made between the potency and cross-reactivity of the antibodies against vasopressin and oxytocin in immunofluorescence and radio-immunoassay (see below). Important differences between the results characteristics of the antibodies in these two procedures were observed. This finding will be elaborated, concerning the underlying mechanisms and the practical implications. This in terms of antibody selection for each technique and with respect to possible problems in interpretation of clinical data, *i.e.*, in autoimmune diseases.

b. *Immunoperoxidase technique (light and electron microscopy).* Immuno-electron microscopy seems the only way to establish a) whether a neuro-endocrine cell synthetizes, and not only stores, one or more hormones, and b) at what sites the hormone is stored and released. Therefore, an immunoperoxidase procedure has been developed based upon the experiences with the immunofluorescence technique. Using light microscopical techniques the optimal conditions for this "sandwich" technique were determined in hypothalamic and neurohypophyseal tissue. The localization of neurohypophyseal hormones in the hypothalamo-neurohypophyseal system and suprachiasmatic nucleus of Wistar and Brattleboro rats being homo- or heterozygous for diabetes insipidus have been published. The results obtained with this technique fully agreed with those obtained with immunofluorescence microscopy.

Based on these light microscopical data, an immunoelectron microscopical procedure on ultrathin sections was developed using hypophyseal tissue. Fixation in 4% formalin, during 24 hours resulted in a reasonable morphology and a good preservation of the antigenicity of the tissue. Addition of 1% tannic acid to the fixative improved the morphology; *i.e.*, some organelles like plasma membranes and collagen fibres were better

preserved. However, this compound could not be added for more than 2 hours because otherwise the background staining of the neurosecretory granules became too high.

Other improvements of the technique were: a) absorption of the second antibody to liver and brain homogenates prior to use in order to remove background staining and b) incubation with diamobenzidine in dark conditions.

After incubation with the antibodies, the reaction product was found almost exclusively within the neurosecretory granules, while the controls showed no reaction product whatsoever. Problems were encountered, however, by aspecific binding to the embedding medium. Therefore the unlabeled antibody enzyme method is performed at present with good results using peroxidase-antiperoxidase complexes (PAP) that were generously supplied by Dr. L. Sternberger (USA). Current research is directed to the specificity of oxytocin and vasopressin antibodies at the electron microscopical level.

This theme forms a part of the thesis of F. W. van Leeuwen.

Theme 2: The role of the fetal hypothalamus and pituitary in intrauterine growth (supported by the foundation "De Drie Lichten")

In human newborns a close relationship exists between low birth weight and high perinatal mortality.

In collaboration with the university clinic of Obstetrics and Gynaecology, evidence was obtained that in human and rat fetus an intra-uterine growth spurt exists (respectively 16–24 weeks and day 19 of pregnancy) and that the hypothalamo-hypophyseal area was essential for this spurt. After systematical injection of many compounds of hypothalamo-hypophyseal origin directly into rat fetuses, only α-MSH appeared to be able to stimulate intra-uterine growth. In order to investigate whether this was only a pharmacological *effect* of exogenous α-MSH or also a *function* of the endogenous hormone, antibodies against α-MSH were raised in rabbits. By the application of very regular immunizations and bleedings and the use of *i.p.* plasmaphoresis, a very large amount of potent antibodies was made available. In the first place we have determined by immunofluorescence whether α-MSH was present in the fetal rat pituitary during the intra-uterine growth spurt. Therefore, cross-reacting components against ACTH (1–24) were removed from the plasmas. α-MSH appeared in the fetal pituitary on day 18, the day before the growth spurt started. The course of the amount of fluorescence in the intermediate lobe has been quantified from day 17 of pregnancy up to 6 days postnatally.

In collaboration with Dr. F. J. H. Tilders (Dept. of Pharmacology, V.U., Amsterdam), extracts have been made of rat fetal pituitaries and bio-assay of MSH-activity was performed. Also with this technique MSH appeared in the pituitary on day 18. In addition a significant negative correlation was found between fetal body weight and pituitary MSH

content on day 19, which supports the idea of a causal relationship between these two data.

Using purified anti-α-MSH that was injected directly into rat fetuses, the endogenous α-MSH was bound. This caused a decrease in intra-uterine growth. These experiments show that α-MSH (for which no physiological function was known in mammals) is essential for normal intra-uterine growth. In order to determine whether MSH is also involved in intra-uterine growth in the human, hundreds of umbilical plasma samples of human newborns and their mothers have been collected in the past year in collaboration with Kloosterman's clinic. In collaboration with Dr. Tilders and Dr. A. Thody we are to determine whether a correlation exists between the blood levels of the various kinds of MSH and birth weight in human. In addition some human fetal and maternal pituitaries were collected. Preliminary studies indicate the existence of α-MSH in these human fetal and maternal pituitaries which is against the general opinion in the literature. The study of the human fetal pituitaries (immunofluorescence and electroforesis) may also indicate which of the various MSH compounds would be of importance during a particular stage in human development.

Theme 3: A possible role of the pituicytes in the neurosecretory process of the rat hypothalamo-neurohypophyseal system (HNS)

The fate of release residues, especially membranes of hormone containing vesicles, within the neurohypophysis is still a point of speculation. It is suggested that the neurohypophyseal glial cells—pituicytes—are involved in this removal, since during changed conditions of the HNS an inverse relationship exists between the axonal neurosecretory material content and the amount of lipid droplets in these cells. Lysosomal activity might be involved for the partial break-down within the nerve terminals and the final catabolism within the pituicytes. By microchemical procedures we have shown indeed an increased lysosomal enzyme capacity for the neural lobe under various conditions that activates the HNS, but section-histochemistry failed in localizing these responses.

Subsequent work on isolation of axons and pituicytes from as little as 1.5 mg rat neurohypophyseal tissue (3 neural lobes) was started therefore and this year optimalized up to a finally standardized procedure. It comprises trypsinization followed by dissociation through sieves of subsequently 200 and 50 μ mesh value and finally by microcentrifugation with a 10/14/22% ficoll gradient in which the dispersed tissue was brought as the 14% layer. Characterization was performed by protein, DNA, lactate dehydrogenase and oxytocin bioassays as well as by light microscopy, oxytocin-immunofluorescence and electronmicroscopy. The pinched-off axonal elements—neurosecretosomes—(interphase 14/22) and pituicyte enriched fractions (pellet) contained ultrastructurally fairly intact elements and their relative purification was about 4 on the basis of oxytocin/DNA i.e., axon/cell ratio.

Fractions isolated after 6 days of water deprivation and 9 to 10 days of lactation were characterized in a similar way, while in addition acid phosphatase histochemistry and microchemistry was performed. The enzyme appeared to be more active within the pituicytes than in the nerve endings, but for the neurohypophysis as a whole the activity appeared to be rather equally distributed between axonal and pituicyte compartment. The activity increase was found to be localized in both elements, but quantitatively mainly present in the neurosecretosomes. The lysosomal response was observed both in neurosecretosomes and pituicytes, but was quantitatively mainly present in the pinched-off axonal endings. The response in both compartments support the postulated final catabolism of release residues by the pituicytes.

The uptake of materials originally composing the neurosecretory vesicle membranes by pituicytes, however, has still to be proven and will be studied by radioactive lipid labeling *in vivo* and subsequent isolation of the pituicyte and nerve ending fractions as has been developed last year.

This theme will form part of the thesis of G. J. Boer (Febr., 1976).

Theme 4: Radioimmunoassay of hormones (supported by ZWO)

Hormone assays of vasopressin, oxytocin and MSH are essential for many aspects within the project "neuroendocrinology". Some problems could be solved by bioassay, e.g., of pituitary levels. The very low levels of these hormones in body fluid (blood, urine, cerebrospinal fluid and amniotic fluid) make the use of radioimmunoassays absolutely necessary. Starting at the beginning of 1975, a radioimmunoassay set-up was installed, based on the extensive experiences obtained within the Rudolf Magnus Institute (Utrecht). Measurement of 0.25 pg vasopressin and 1 pg oxytocin per tube is possible at present. Although the oxytocin assay has been improved last year, there are certainly possibilities to make this determination even more sensitive. The extraction of these two hormones from body fluids has been developed for vasopressin to a higher degree, but needs still some improvements for the oxytocin assay.

In collaboration with a guest worker Prof. Dr. J. V. Milligan (Canada) and the computer department, a computer program was written and tested that has been designed to read and analyse paper-tape output from gamma counter. Linearizing transformation of the standard curves was obtained by "log" and "logit" transformations according to Rodbard (NIH, USA). Calculations for recovery estimation, final concentrations and dilutions were added. This program enables us to receive the assay values, plot and characteristics of the standard line within 10 minutes. This means a time saving of one day per assay.

The vasopressin blood assay was tested e.g. by studying the effect of histamin, a putative hypothalamic transmitter, on bloodlevels in rat. A

time-effect and dose-effect relationship has been described after *i.p.* injection of this drug. Intracerebroventricular injection of histamin also caused vasopressin release into the blood, even with lower doses and quicker than by *i.p.* administration, which may indicate that histamin has an important physiological transmitter function in the hypothalamus.

Oxytocin and vasopressin determinations have also been performed for the Dept. of Pharmacology (V.U.) and the university clinic of obstetrics. For Dr. F. J. H. Tilders the assays were performed in the framework of his investigation of the role of the neurohypophyseal hormones in the intermediate lobe function. The results are mentioned in his thesis.

Bloodsamples of pregnant patients having diabetes insipidus versus normal pregnant women and control persons were performed. These were the first combined oxytocin and vasopressin blood assays in patients in the Netherlands. The results were compatible with the diagnosis "hereditary hypothalamic diabetes insipidus". Furthermore assays will be performed in patients having toxaemia of pregnancy in which vasopressin is suggested to be a causal factor.

A radioimmunoassay for various components having MSH activity will be started within a few months, in collaboration with Dr. F. J. H. Tilders (Dept. of Pharmacology, Free Univ., Amsterdam) and Dr. A. J. Thody (New Castle, U.K.).

Part of this theme is a thesis subject of J. Dogterom.

Theme 5: Neuropeptides and memory

As has been shown by the group of Prof. de Wied (Utrecht), lack of vasopressin causes memory deficiency in rats. Our interest in this phenomenon is how vasopressin is reaching the brain cells (via the peripheral circulation, cerebrospinal fluid) and where in the brain this hormone is acting.

Therefore, vasopressin levels in body fluids are studied during behavioral tests i.e. passive and active avoidance behavior in rats. The behavioral experiments are performed in De Wied's laboratory.

Extensive studies performed last year did not reveal any correlation between plasma vasopressin and behavioral parameters. However, Van Wimersma Greidanus *et al.* (1975) showed that antibodies injected into the cerebroventricular space, induced the behavioral signs of vasopressin deficiency. This indicates that vasopressin is released into the ventricle and reaches in this way its target sites. Therefore, vasopressin is now assayed in cerebrospinal fluid of rats.

The presence of vasopressin in cerebrospinal fluid has been demonstrated already in rat, dog and human by us. A possible influence of anaesthesia on these levels is studied presently in the rat and the dog. Techniques to obtain cerebrospinal fluid samples from the conscious and unrestrained rat will be developed next year.

A comparative study of vasopressin levels in blood, urine and cerebrospinal fluid in human, rat and dog, under basal and various experimental conditions will be completed this year.

Because intraventricular administration of antibodies against vasopressin causes memory deficiency in rat, a study was initiated on the question whether the antibodies are staying within the ventricle and bind vasopressin here or penetrate into the brain. Using fluorescence of FITC-labeled immunoglobulins, we found that these compounds could not be detected longer than 15 minutes, while indications were obtained of uptake by the ependym. Using immunofluorescence, we will study whether anti-vasopressin is concentrated in any particular brain region. In addition possible receptor sites for vasopressin will be traced by incubating brain sections in a medium, containing this hormone, and subsequent immunolocalization procedures.

This theme will form part of the thesis of J. Dogterom.

Theme 6: The relation between the hypothalamo-neurohypophyseal system (HNS), lactation and parturition

The HNS synthetizes and releases oxytocin and vasopressin. One well established function of oxytocin is the ejection of milk from the mammary gland in lactating animals. The release of oxytocin in the lactating rat occurs intermittently in pulses. These pulses are preceded and probably induced by burst-like increases in the HNS neuronal firing rate. The study of the milk ejection phenomenon in the rat by means of intramammary pressure recording has been performed until recently under anaesthesia. Lincoln and Boer (1974) have developed a telemetric way of intramammary pressure recording in the conscious and unrestrained rat. In this way a study of environmental influences upon lactation and oxytocin release has been made possible. Preliminary data concerning the inhibitory effect of stress have been described. These data, however, have to be extended, while the reproducibility of the technique has to be improved. In the next year other conditions that affect or are likely to affect oxytocin release (water deprivation, alcohol administration, hereditary diabetes insipidus), will be studied. Additional information will be obtained so for the study of parturition during the same conditions, published by Boer, Boer and Swaab (1974). In addition it is important for the understanding of the oxytocin release mechanism to see how a regular all-or-none type release, as during lactation, reacts upon afferent stimulation or inhibition.

As far as parturition is concerned it is not certain, whether or how oxytocin plays a role in the rat (see Boer, Boer and Swaab, 1974). There are data obtained by our group, that point to a higher synthetizing activity and even a higher release of oxytocin at this stage. Furthermore Boer, Lincoln and Swaab (1975) were able to induce uterine contractions

in the conscious rat by electrically stimulated release of endogenous posterior lobe hormones and even to accelerate expulsion of pups. On the base of the neurophysiological data obtained in lactating rats, it seemed worthwhile to study HNS neuronal activity during labour. The first question to be asked was: do such bursts in firing rate as found during suckling also occur during parturition? From the already available data, it is clear, that bursts in firing rate do not occur neither before nor during the expulsion of pups, uterine contractions are present. Moreover, no obvious time relationship is seen at all between neuronal activity of *in vivo* identified HNS cells and uterine contractions, measured as intra-uterine pressure changes. Minor changes will be detected or excluded by computer analysis (developed in co-operation with Prof. J. V. Milligan, Canada). There are, however, changes in mean discharge rates of cell-firing and excitability related to the reproductive stage. The highest firing rates were found during parturition, and to a lesser extent in the oestrogen stages, whereas lower neuronal activity exists in the non-oestrogen stages. Hardly any of the so-called quiescent cells were found during parturition in contradiction to normal cyclic females studied by us and others. These changes are indicative for an increased hormone release during labour.

Oxytocin blood levels during all tested conditions will be measured by radioimmunoassay, developed in the neuroendocrinology group. A start is made to determine oxytocin blood levels during water deprivation.

These themes will form part of the thesis of K. Boer.

Theme 7: Thyroid hormones and brain development

Thyroid hormones are essential for normal brain development. For the action of thyroid hormones sufficiently iodine uptake via drinking water or food is necessary. Since in large areas of the world the food soil is iodine deficient, large populations have disturbed thyroid function and therefore (as has been shown by Prof. Dr. A. Querido, Leiden and collaborators) a disturbed mental capacity which has most probably a permanent character. Collaboration with Prof. Querido has lead to a monography (Querido and Swaab, 1975) and to the possibility of future collaborative animal experiments on brain development during iodine deficiency, which will include biochemical parameters for brain development and studies on nerve cell arborization (compare Uylings).

Students/guest workers

F. Nijveldt (student pharmacy – Immunolocalization of vasopressin and oxytocin in the hypothalamus of the rat, 1974/1975. *Treatise:* The physiology and pathology of somatostatin.

J. Brugge (student biology) – Quantitative electronmicroscopy of dispersed neurohypophyseal cellular elements, 1974/1975.

G. Snoek (student biology) – Radioimmunoassay of ACTH 1–10. Accompaniment by J. Dogterom on the Rudolf Magnus Institute (Utrecht).

Drs. M. Visser (W. G., gynaecologist in training) – The role of α-MSH in intra-uterine growth, 1975/1976.

A. Bernards and P. Liebrechts – Characterization and purification of antibodies against ferrodoxin (in collaboration with Lab. of Plant Physiology).

Prof. Dr. J. V. Milligan (Canada) – Computer analysis of radioimmunoassay and action potentials.

ELECTRON MICROSCOPIC INVESTIGATIONS ON THE MORPHOLOGY OF ENDOCRINE STRUCTURES IN THE MAMMALIAN PINEAL GLAND IN RELATION TO THEIR FUNCTION

Participants

Dr. H. J. Romijn (biologist, 80%)
Drs. P. Pevet (biologist, p.m., from 1.10.1975)
(for Progress Report see Prof. Dr. J. Ariëns Kappers)
P. S. Wolters (technician, 65%)
M. T. Mud (analyst, 100%)
S. W. Lust-Bosboom (secretary, 10%)
Prof. Dr. J. Ariëns Kappers (advisor)

From clinical observations is known that pineal tumors may be of considerable influence on the development of puberty. Laboratory studies have shown that the mammalian pineal is an endocrine organ having among others an antigonadotropic function. So far, the exact nature of the gonadotropic factor is not exactly known, despite extensive research. On the one side biochemical studies point to indoleamines (serotonin/melatonin), on the other to polypeptides. In addition, recent investigations suggest functional relationships between the pineal and (fetal) growth, the (para)thyroid glands, the adrenals, blood pressure and body temperature.

Currently, pineal research has mainly developed in the biochemical direction. Neuroanatomical studies form the base of the present project.

Theme 1: In vivo studies

In the past years our project group studied the structure and innervation of the rabbit pineal gland under normal and experimental conditions using light and electron microscopy. It appeared that, in the rabbit pineal, a cortex and a medulla as well as two types of pinealocytes, light and dark ones, can be distinguished. Cell structures expected to play a significant role in pineal hormone synthesis were described. Moreover, it was demonstrated that the pineal is innervated by both the sympathetic and the parasympathetic nervous system.

Surgical sympathectomy of the pineal organ by bilateral extirpation or decentralization of the superior cervical ganglia caused characteristic ultrastructural changes in the light pinealocytes which were similar to those observed after exposure of rabbits to continuous illumination. On the basis of data from pineal biochemistry it was supposed that these changes had a common origin, viz. the lack of noradrenaline, the pinealotropic neurotransmitter. The hypothesis was put forward that the smooth endoplasmic reticulum in the light pinealocytes is somehow involved in pineal indoleamine synthesis.

This year the pineal gland of rabbits chronically injected with sympatholytic, parasympatholytic and serotonin-synthesis inhibiting agents was studied by electron microscopy. The results obtained after administration of p-chlorophenylalanine and p-chloroamphetamine support the hypothesis proposed earlier that the smooth endoplasmic reticulum in the light pinealocytes is involved in indoleamine synthesis. The administration of either one of the sympatholytic agents, 6-hydroxydopamine or α-methyl-p-tyrosine, induced typical fine structural changes corresponding to those observed after surgical sympathectomy.

Theme 2: Quantitative assessment

It is generally known from pineal biochemistry that the serotonin/melatonin content as well as the ethanol-soluble lipid fraction in the mammalian pineal gland follows a diurnal rhythm. In an attempt to demonstrate such a diurnal rhythm ultrastructurally a special parameter of Golgi secretory activity in the light pinealocytes was analysed electron microscopically by counting during a day/night cycle the number of dense-core vesicles present a) in the direct vicinity of Golgi systems and b) in the terminals of cell offshoots. The results obtained evidence for the existence of a diurnal rhythm in the number of Golgi dense-core vesicles in both areas being high at noon (a) and in the evening (b) respectively.

Theme 3: In vitro studies

Aim of this project is to interpret the highly differentiated ultrastructure of the mammalian pinealocyte in a *functional sense*. Firstly we wish to localize at the subcellular level the site of the serotonin/melatonin synthesis, and, secondly the exact sites of synthesis and secretion of the anti-gonadotropic hormone(s). In close collaboration with Dr. J. Peters (Department of Pharmacology, University of Amsterdam), an organ culture of the rat and rabbit pineal tissue was set up for this purpose. At the moment rat and rabbit pineal tissue can be kept in good condition with maintenance of the normal ultrastructure for at least 8 days (see student-report A. J. Gelsema).

Starting from the principle that physiological changes are not seldom reflected in morphology, pineal tissue was cultured after addition of,

respectively, p-chlorophenylalanine, noradrenaline/cyclic AMP, pilocarpine or nialamide to the culture medium. In a second experiment, a self-made tryptophan deficient medium was used to which tryptophan, 5-hydroxy-tryptophan or serotonin, partly combined with p-chlorophenylalanine, was added. The tissue is examined electron microscopically at present.

Together with Dr. M. Karasek, a Polish guest worker, and in collaboration with Dr. F. W. G. Flight (Dept. of Electron Microscopy, University of Utrecht) technical preparations are being made for electron microscopic autoradiography in pineal tissue cultures. The aim of these experiments is the subcellular localization of the serotonin/melatonin synthesis in the mammalian pineal gland by culturing rat and rabbit pineal tissue in the presence of 5-hydroxy (^{3}H) tryptophan or (^{3}H) serotonin. These experiments are planned for January of 1976.

Theme 4: Service

The department of electron microscopy has a central position at the Institute. The need of other sections for careful electron microscopic accompaniment of their various projects necessitates daily service by all co-workers of our section. This includes aspects such as fixation, embedding, ultrathin sectioning and assistance with the use of the microscope.

Service was rendered to Dr. M. A. Corner, Dr. R. Baker, Dr. A. B. Oestreicher, Drs. G. J. Boer and to Drs. P. Pevet.

Students/guest workers

A. J. Gelsema (medical student), 9 months, Preliminary electron microscopic investigations of rabbit pineal organ culture.

Dr. M. Karasek (Lodz, Poland), 6 months, Electron microscopy of rat pineal organ structure.

THE NEUROLOGICAL BASES FOR MOTIVATIONAL PROCESSES IN REPRODUCTIVE BEHAVIOR

Participants
Dr. N. E. van de Poll (psychologist, 100%)
B. Kouwenhoven (cand. ass. psychology, 50%)
J. G. M. van Oyen (cand. ass. psychology, 50%)
M. M. Smidt (secretary, 45%)

In this project the concept of "motivation" is studied within the framework of a detailed analysis of various aspects of reproductive behavior, and its neural substrate. The investigations are aimed at the nature and localization in the brain of processes lying behind sexual motivation, especially in so far as these are influenced by hormonal factors. Moreover, the consequences of different but related motivational factors controlling overt behavior, as for instance aggression, are analysed. The

sex differences in behavior and the organization of the central nervous system by hormonal factors early in development is an important factor in this respect and provides a good instrument for the investigation of motivation.

This project originated from earlier work on self-stimulation in relation to sexual behavior and experiments on sexual behavior in rats, initiated by Drs. H. van Dis in co-operation with Prof. K. Larsson (Götheborg, Sweden). The work on hormonal aspects of aggressive behavior and its relation to sexual behavior was started with personal and financial support of the "Beleidsruimteproject, Hersenen en Gedrag: Agressie".

H. van Dis fulfilling part-time an appointment in the clinical department of the psychological faculty of the University of Amsterdam, this year devoted full attention to the possible clinical implications of the NCIH motivation project. His clinical contribution from outside the Institute may become of great value for this project in the future.

Theme 1: Hormones and sexual motivation

Our investigations thus far have been aimed at the study of the activational and organizational role of gonadal hormones upon homo- and heterotypical sexual behavior in male and female rats. In male rats, feminine sexual behavior patterns could be activated by estrogen treatment. Moreover, factors affecting the levels of male sexual activity, i.e., castration, sexual exhaustion and congenital sexual inactivity were shown to decrease the levels of hormone induced feminine sexual behavior in these males. In contrast to earlier findings in literature, progesterone proved to facilitate estrogen induced feminine sexual behavior in males.

The "organization" of the sexual behavioral responses in male and female rats by androgen during the perinatal period was studied in relation to the activation of sexual behavioral responses by gonadal hormones in adulthood. This first series of basic experiments is finished (Ph.D. dissertation Van de Poll) and the results are currently being prepared for publication.

Research will next be directed to the development of new test situations in which motivational elements of behavior such as the preference for specific stimuli in the environment are studied as behavioral parameters. Hereby a further analysis of the nature of the behavioral effects of hormonal and lesion studies will be served, and it will possibly become a starting point for extrapolation of the results to man.

The phenomenon of sexual exhaustion, occurring in male rats specifically affecting the motivational component of behavior, has also been studied. Testicular androgens appeared to be unessential for processes occurring during recovery from exhaustion. These results too are now being prepared for publication.

Hormonal products are among the main factors influencing sexual motivation. Relatively little knowledge exists about the hormonal

influences upon other sex differences of behavior or influences of behavior upon hormone production and maturation. The continuation of this project in co-operation with the project "Neuroendocrinology" would open fruitful possibilities in this respect.

Theme 2: The localization of brain centers of sexual behavior

Bilateral radiofrequency lesions in the medial preoptic and anterior hypothalamic areas appeared to abolish masculine sexual behavior in male rats. Feminine sexual behavior was slightly facilitated by lesions in the anterior hypothalamus, however, suggesting that in the male rat two distinct systems are present at this level. These results are currently being prepared for publication. New experiments in which electrodes are bilaterally permanently implanted in these regions in order to combine studies of electrical stimulation, self-stimulation and lesion effects are in progress. The results promise to increase our understanding of hypothalamic functioning by giving the opportunity to correlate the behavioral effects of electrical stimulation, self-stimulation data and the results of lesions at the same place in the brain.

Further experimentation will be directed to the localization of masculine and feminine motivational centers in the female rat and the analysis of behavioral changes that are caused by destruction of these centers. These experiments will be planned on base of a co-operation with the anatomical department of the University of Nijmegen (Dr. A. H. M. Lohman). A new series of experiments will be aimed at a more detailed analysis of the behavioral changes resulting from these experimental treatments (see theme 1).

Experimental manipulation of feeding conditions, hormonal factors or social conditions, for instance social isolation or overcrowding, lead to specific changes in social behavior. A combined study of these aspects of behavior and the underlying endocrinological and morphological changes by the methodology and techniques of the projects "Neuroendocrinology" and "Neural Plasticity" at this Institute will create deeper understanding of both the concept of motivation as well as the aspects of neural plasticity and neuroendocrinology itself.

Self-stimulation and sexual motivation

Electrical stimulation of the preoptic area and surrounding structures appeared to have strong reinforcing characteristics. Self-stimulation in these areas in male rats is dependent upon the hormonal state of the animal: castration decreases the rates of self-stimulation, subsequent hormone treatment reinstates this behavior. A further study of electrical self-stimulation of these structures will gain more information about the centers localized in theme 2 and will create deeper understanding of the nature of the motivational processes and the influencing factors.

With aid of a new self-stimulation unit, developed in this laboratory, the above line of experiments is being followed. An interface, being developed at this moment will allow a combination of the stimulators with the Grason-Stadler Programming units (theme 3), makes the self-stimulation data accessible for computer analysis and creates possibilities for more advanced experiments on this matter.

Self-stimulation sites in the preoptic and anterior hypothalamic regions of male and female rats are more precisely being localized, and the influence of specific gonadal hormones will be investigated. This study will be combined with the other techniques and experiments of theme 2.

Theme 3: Sex differences and hormonal factors in learning processes

In the human, sex differences in behavior are well-documented. It is unclear, however, whether these differences are predominantly caused by cultural and educational variables or are predetermined by individual differences in "learning potential". Experiments on animals might illuminate this and provide further insight into the behavioral consequences of the "organization" of the CNS caused by hormones during development (theme 1).

Pilot experiments have been finished in the course of this year and definitive experiments on the role of gonadal hormones on learning behavior are soon to be started. These learning experiments are programmed with aid of Grason-Stadler programming units (1200-systems). An interface was developed in this laboratory to make the behavioral data accessible for computer analysis in combination with the "White-system" (theme 4).

Interest is focused on the differences between active and passive avoidance performances. Male rats are known to learn faster in inhibitory conditioning (passive avoidance). In active avoidance procedures, however, females do better than males. This reversal is also found in the extinction of the responses. Critical variables in this reversal and the role of gonadal hormones will be investigated. For details of the learning experiments Drs. D. Fuldauer of the Clinical Department of the Psychological Faculty of the University of Amsterdam, is consulted.

Theme 4: Sexual motivation and aggressive behavior

The relation between sexual and aggressive behavior and the role of gonadal hormones in the regulation of aggressive behavior is also being studied in the scope of this project. Sex differences of behavior and the specificity of the behavioral effects revealed in the study of sexual motivation in theme 1 and theme 2 is the chief interest at this moment. Aggressive and sexual behavior is studied in the S-3 strain, a more aggressive strain than the Wistar rats.

Preliminary studies were done to develop an adequate set of behavioral parameters. Moreover, an electronic keyboard system (White-system, built

in this laboratory) makes the behavioral data observed from a videotape accessible for computer analysis.

Two experiments were executed in 1975 and are being analysed at this moment:

1. In a methodological study aggressive behavior of male S-3 rats is studied after 24 hours of isolation or after 1 hour of isolation. The aggressive behavior patterns against another stimulus male are compared. The raw data are to be analyzed by statistical treatment but casual observation indicates that 1 hour isolation induces sufficient levels of aggression to allow further experimentation on this subject.

2. Aggressive behavior of female S-3 rats and the influence of testosterone administration is being studied. In this experiment the animals are tested against ovariectomized females or against ovariectomized females treated with testosterone, in order to analyse factors from the stimulus situation. In this experiment special attention had to be payed to the development of a set of behavioral data relevant for aggressive behavior in the female. Moreover, masculine and feminine sexual behavior was studied in the course of this experiment. The results are further analysed. Tentative analysis leads to the following conclusions:

a. The occurrence of isolation induced aggression is a common phenomenon in the ovariectomized female S-3 rat.

b. The latencies of the aggressive parameters of the observed behavior seem to shorten when the animals are being treated with testosterone.

c. The nature of the stimulus situation, a female treated with testosterone or not predetermines the levels of aggressive behavior especially by inducing masculine sexual behavior patterns.

Investigations for the next year will include a further elaboration of sex differences in aggressive and submissive behavior, and the study of hormonal factors in the regulation of these two kinds of behavior. Moreover, specific changes in masculine sexual motivation will be studied in relation to changes in aggressive behavior. The experiments on aggressive behavior are planned and co-ordinated with the "Landelijke agressie werkgroep".

Student

A. Zuiderwijk (student biology), Aggressive behavior in the female rat.

CLINICAL PSYCHOBIOLOGY

Participant
Drs. H. van Dis (med. drs. and psychologist, 100%)

In the past years within the project "Motivational processes of reproductive behavior" interest increased in the relevance of basic "brain and behavior" studies for clinical psychology and psychiatry. This was

made explicit by a part-time appointment in the department of clinical psychology of the University of Amsterdam in 1971.

In 1975 the development of automated registration and computer analysis of EEG, EOG, EMG, ECG, respiration and skin resistance measures was completed. This progress was necessary for an intensive study of three research themes.

Sexual arousal in human subjects

The idea of studying human sexual behavior directly stemmed from the project "Motivational processes of reproductive behavior". In our animal studies of feminine and masculine behavior discrete aspects of sexual behavior could be separated behaviorally and differentially be influenced by neural, hormonal and pharmacological manipulations (e.g., approach behavioral elements, consumatory behavioral elements, refractory phenomena).

In human experimentation an adequate behavioral descriptive system for the study of sexual behavior is lacking. A growing amount of work was directed to its concomittant autonomic changes. Our approach to the study of sexual behavior was mainly centered upon the electro-encephalographic and autonomic changes during the several phases of sexual arousal, in search for parallel aspects of sexual behavior as were conceptualized in the animal studies. Most promising at the moment is the identification of refractory phenomena both in the human male and the human female. In the future the studies will include hormonal effects on these refractory phenomena, thus more closely interacting with the project "Motivational processes of reproductive behavior".

Biochemical and psychophysiological aspects of anxiety in patients with anxiety neurosis or phobic neurosis and in control subjects

In the department of clinical psychology phobias are studied from different psychological perspectives. In this project psychophysiological registrations have been gathered from 200 phobic patients. The results are (a) to identify diagnostic criteria for the prediction of success for several kinds of therapy (with promising results after analysis of the first 50 patients) and (b) to identify somatic factors underlying phobic symptoms. Approximately 50% of the phobic patients appeared to present the hyperventilation syndrome (panic attacks together with somatic symptoms due to overbreathing).

The psychophysiological concomittants and pharmacological regulation of subjective and objective hyperventilation symptoms was studied in non-patient subjects. Furthermore several experiments have been undertaken on the pharmacological induction of subjective complaints mimicing anxiety and somatic anxiety equivalents (such as palpitations, paresthesia, breathlessness, dizziness, etc.). The first study on the effect of sodium lactate infusion has been completed.

Sleep disorders in patients with anxiety neurosis and phobic neurosis

In the study of the relation between sleep and psychopathology much work has been published on the polygraphic sleep analysis in depressed patients, schizophrenic patients and other hospitalized psychiatric patients. Data on the sleep of extremely anxious patients are lacking although these could be of theoretical relevance to the study of sleep in relation to psychopathology.

Systematic interviewing of phobic patients with respect to different psychobiological functions has focused attention on several possible underlying biological processes. Frequent complaints of sleeping disorders in phobic patients and patients with recurrent panic states initiated the polygraphic studies of sleep patterns in anxious patients. This line of research has been started recently, after a 2-year period of preparation.

SECTION OF ETHOPHYSIOLOGY

Participants
Drs. A. P. van der Mechè (biologist, 100%)
Drs. B. Bermond (psychologist, 100%)
Drs. J. P. C. de Bruin (biologist, 100%)
Dr. A. Miodonski (M.D., biologist, 10%)
V. Nolten (analyst, 100%)
S. W. Lust-Bosboom (secretary, 20%)

The project deals with brain and behavior, i.e., the function of telencephalic and diencephalic areas in memory (theme 1) and aggression (theme 2).

Theme 1: The neural and chemical substrate for learning and memory in the goldfish (Carassius auratus L.)

The telencephalon of fish plays an important role in the natural behavior of fish. Literature data suggest that it also plays a role in learning behavior.

To complement the results of lesion experiments in the telencephalon on natural behavior, experiments have been performed to study the effects of telencephalic lesions on learning behavior. The experimental set-up is based on barrier-crossing behavior of goldfish in a shuttle-box. The results of the telencephalic lesion studies on active avoidance conditioning showed that after extirpation of the telencephalon spontaneous barrier-crossing disappeared. However, both acquisition and retention of the active avoidance conditioning response were unimpaired. These data necessitate a reconsideration of the current hypothesis in literature according to which the normal function of the telencephalon is to facilitate the processing of information.

A precise histological control of the brains after telencephalic extirpation revealed that part of the telencephalic/diencephalic peduncle, i.a. consisting of the nucleus preopticus and the nucleus entopeduncularis remained

present. We are of the impression that the preoptic region is unchanged, but that the nucleus entopeduncularis shows a change in structure. Further research has to be done to confirm this and to evaluate possible behavioral consequences of damage to these areas.

Besides the experiments with negative reinforcement, experiments have been performed with positive reinforcement. Although the experimental procedure is more difficult for the fish, various animals show a significant increase in response frequency during the light stimulus at the end of the conditioning phase. However, this is also the case under a pseudo-conditioning control procedure, in which reinforcement is not contiguous with the light stimulus. Telencephalic removal results in a nearly complete disappearance of barrier-crossing. Further research is needed to determine whether this result is due to the disappearance of spontaneous barrier-crossing after telencephalic extirpation, as was stated above. An analysis of the influence of light presentations during the first conditioning-session reveals that these presentations tend to decrease the barrier-crossing behavior as compared with barrier-crossing behavior without light presentations during the preceding days.

Future research

a. *Neuroanatomical and behavioral aspects.* The above mentioned results of total telencephalic ablation indicating a facilitating telencephalic influence on barrier-crossing, but no impairment, neither on acquisition, nor on retention of an active avoidance conditioning, lead to the following steps:

- to localize a region which, if lesioned, will impair negative reinforcement;
- to localize a telencephalic region essential for spontaneous barrier-crossing behavior;
- to study if a lesion in this place will impair conditioning with positive reinforcement;
- neuroanatomical degeneration experiments to study degenerative effects on the remaining brain (see also theme 2).

b. *Neurochemical and behavioral aspects.* To study the chemical alterations in the brain due to experience, correct control experiments are often problematic. Our experimental set-up with goldfish will be advantageous for these studies, because: 1. various learning processes can occur in the same experimental set-up (experiments with both negative and positive reinforcement), 2. split-brain preparations are possible.

During the previous years much experience has been gained on item 1. Considering item 2 the commissures at telencephalic, diencephalic and mesencephalic levels can be cut and lead to a split-brain preparation. Goldfish have the advantage over mammals that the optic nerves show a complete crossing to the contralateral side. Covering one eye in a

split-brain goldfish prevents visual information to reach the contralateral side. Conditioning of such animals will most probably lead to differences in the two brain halves: an experienced one, and a naive one serving as the control.

A study is planned using split-brain goldfish in which eye-caps will be attached to both eyes, one transparant and one untransparant. The first aim will be a behavioral control of the effectiveness of the split-brain preparation. This will be followed by a study of the differences between the two brain halves, using autoradiographical (and other) techniques (a preliminary introduction has been presented in the 1976-project planning).

The research is regularly discussed with Dr. J. Segaar, former head of the section of ethophysiology. Further contacts exist with promotor, co-promotor and co-referent (Prof. Dr. J. Ariëns Kappers, Prof. Dr. J. J. A. van Iersel, Prof. Dr. P. Sevenster; Laboratory of Zoology, University of Leiden). For the chemical approach contacts exist with Prof. Dr. P. Borst (Laboratory of Biochemistry, University of Amsterdam). Inside the Institute the main contacts are with the sections of Neuroanatomy and Neurochemistry.

Theme 2: Neural and hormonal regulation of aggressive behavior in the siamese fighting fish (Betta splendens) and in the S-3 rat (Rattus norvegicus)

Aggressive behavior has been associated with brain areas of telencephalic, diencephalic and mesencephalic origin. Preliminary data suggest that parts of the limbic system (s.s.) and the hypothalamus play an important role, both in teleost fish and in mammals.

In fish our research is focused on the dorsal and preoptic area of the telencephalon, a brain region which is the evolutionary predecessor of the limbic system, and which in teleost fish regulates the expression of aggressive and reproductive behavior. In the rat the preoptic and adjacent anterior hypothalamic areas are the focus of interest, as it is the region which is considered to be an important target area for androgens, which influence both aggressive and sexual behavior.

The results of the telencephalic lesion studies in Betta splendens were briefly reported in the 1974 Progress Report. These data have been further analysed and will be published in the thesis of J. P. C. de Bruin. At the same time a review paper is prepared dealing with behavioral effects of telencephalic lesions in teleosts, approached from a neuroethological point of view. In affiliation with the rat studies, the Betta splendens-studies have been extended with a hormonal approach. Both testosteron propionate and 17-α-Methyltestosteron are administered to male Betta splendens and the effects on aggressive, nest building, and sexual behavior are being studied. The main aim of this study is to determine to what extent low levels of these behaviors may be increased by testosteron and to which

degree those behavioral changes are comparable with similar changes resulting from brain lesions as studied before.

In the rat research on aggression was started by developing an ethological acceptable method for inducing intermale aggressive behavior. S-3 rats were finally preferred above Wistar-rats because of their higher levels of "spontaneous" aggression after giving the opportunity to establish a territory. Much effort was spent in determining which behavioral parameters had to be chosen on the basis of their reliability in scoring and their validity as true aggressive parameters. Following these methodological preparations two different approaches have been followed:

a. the effect of castration (depletion of androgens) on aggressive behavior;

b. the effect of lesions in the preoptic-anterior hypothalamic area on aggressive behavior.

The results of the first approach showed a decrease of aggressive behavior. However, this decrease appeared to be much slower and much smaller than literature data suggested. It took a period of 4 months before castration resulted in a statistically significant decrease of aggressive behavior. In contrast to sexual behavior which has completely disappeared four months after castration, intermale aggression may still be very vehement, even one year after castration.

In the second approach the hypothesis, suggested in the 1974 Progress Report was tested. From the results it may be concluded that:

1. large lesions, destroying a major portion of the medial preoptic-anterior hypothalamic region, abolished both male sexual behavior and intermale aggressive behavior. Daily, somatic administrations of high doses of testosteron, for a period of 3 weeks, were unable to reinduce either kind of behavior.

2. Small lesions, destroying minor parts of the same area, did not abolish either behavior, but resulted in a decrease of sexual and/or aggressive behavior. In a careful analysis the neuroanatomical localities of the lesions were correlated with the behavioral changes. These results confirmed the previous hypothesis of a functional division of this brain region: an anterior part which plays a role in aggressive behavior and a more posterior part, playing a role in male sexual behavior. These neural regions are probably the target areas for androgens, stimulating aggressive and sexual behavior.

Our version of White's behavioral registration system, which was developed and built in a close co-operation with both the Electronics workshop and the Computer Section (c.f., their yearly Progress Reports) have proved to be a reliable and time-saving system. Behavioral data can now also be arranged in a transition matrix, thus enabling a more detailed analysis of temporal associations between behavioral parameters; such an arrangement of behavioral data can also be used to submit these data to a factor analysis and other statistical procedures.

The results of the Betta studies have raised many questions concerning comparative aspects of brain and behavior in, e.g., teleost fish and rodents. The telencephalon areas, which have been shown to be important for aggressive behavior, are most probably part of a neuronal circuit which encompasses the hypothalamus as well. A lesion and degeneration study is planned to obtain a better understanding of those circuits; it will be done in co-operation with theme 1 and the neuroanatomy section.

Neuroanatomists often consider a large part of the dorsal telencephalon of teleost fish to be homologous with the mammalian hippocampus. However, notwithstanding the abundance of data on hippocampal functions in learning and memory in mammals, data on the role of the hippocampus in aggression are scarce. Preliminary data show the hippocampus to be of importance in the establishment of hierarchies, while other findings suggest a functional division of the hippocampus in an aggression-facilitating and an aggression-inhibiting area. These latter findings point in the same direction as the results obtained from the Betta studies. The present experimental set-up, from which much experience has been gained on the induction and measuring of aggression in rats, enable a study on the function of the hippocampus for aggressive behavior in rats.

The above stated hypothesis of two separate androgen sensitive areas, one for aggressive and one for sexual behavior, needs further testing. Implantation of testosteron in fully grown rats, castrated at a puberal age, should answer the question of the androgen sensitivity of these areas. However, due to the minimal amount of testosteron to be implanted for successful stimulation and the relatively small size of the neural structure involved, it remains to be seen if this study will also confirm a separation in two areas. Such a differential effect can be shown by an experiment in which two very thin electrodes are implanted in these areas, in one and the same animal. An experiment like this is now possible because Van der Poel and Kruk (State University of Leiden, Dept. of Pharmacology) have developed a method for making and implanting these electrodes.

It has been shown by Thiessen *et al.* that the territorial marking behavior of gerbils is also under androgenic control, while the preoptic-hypothalamic area has been suggested as the androgen receptor site. Similar findings for urine marking in dogs and cats were reported by Hart. It seems possible that this brain area may be subdivided in at least three regions, each acting as a receptor site for androgens and subserving either intermale aggression, male sexual behavior or territorial marking behavior. The first step in solving this question could be taken by an area preoptica-hypothalamus anterior lesion study in gerbils and by correlating the histological data with the change in the three kinds of behavior, i.e., aggressive, sexual and marking behavior as was already done in the rat for aggressive and sexual behavior.

The research of this theme forms part of the activities of the National

Aggression Working Group, co-ordinated by Dr. P. R. Wiepkema (Zoological Lab., University of Groningen). Further contacts exist with promotors, co-promotors and co-referent of the theses of B. Bermond and J. P. C. de Bruin (Prof. Dr. J. Ariëns Kappers, Prof. Dr. N. Frijda, Dr. H. Albrecht, Dr. G. Verberne and Dr. P. R. Wiepkema). The research on fish (themes 1 and 2) is regularly discussed with Dr. J. Segaar, former head of the section Ethophysiology. Within the Institute working contacts exist with the section of Neuroanatomy, Comp. Physiology and the Computer Department.

Students

D. Hermes, biology student at the University of Amsterdam, studied: "Shuttle-box conditioning of goldfish with positive reinforcement".

Mrs. J. van Gestel, biology student at the University of Amsterdam, studied: "The agonistic behavior of the Siamese Fighting Fish, the androgen control and its temporal patterning".

J. Cornelisse and P. Wisman, who obtained histological training for analyst in the Anatomy section, were provided with help and material.

CEREBRAL BLOOD FLOW

Participants
Drs. C. V. de Blécourt (M.D., 100%)
Drs. P. A. de Groot (M.D., 100%)
Ir. J. Smith (advisor)
Dr. J. C. de Valois (M.D. advisor)
M. M. Smidt (secretary, 5%)

This subject is registrated as a project of the FUNGO group "Peripheral Circulation", under nr. 13-26-09.

In the past years, the cerebral blood flow (CBF) in the rabbit has been measured under various experimental conditions, using the method of intra-arterial injection of radio isotopes (^{85}Krypton and ^{133}Xenon). The conditions studied included the effects of vasoactive drugs and anaesthetics on the CBF. As part of a multidisciplinary study CBF has been measured in addition during experimentally induced hypercholesterolaemia. The results of these measurements have been published and resulted in the thesis of J. P. C. Peperkamp.

The following themes are based upon a continuation of this research.

Theme 1: Alteration of CBF in cerebrovascular disease

CBF studies have yield some new data for our understanding of pathophysiology of cerebrovascular diseases. As a basis for clinical investigations in co-operation with the Neurosurgical Clinic of the University of Amsterdam (Head: Prof. Dr. W. Noordebos) an experimental model has

been developed to study the circulatory effects of cerebrovascular disorders. Either by unilateral internal carotid ligation or by induction of arterial spasm some of the most significant symptoms of cerebrovascular disease were simulated. To investigate also the possible influence of different isotopes on the flow measurement itself, CBF was measured with ^{85}Krypton and ^{133}Xenon in the same time in this experimental model. In addition a technique was developed to measure CBF in small volumes of brain tissue by means of ^{14}C-antipyrine.

In these experiments, which are fully in progress, two interesting phenomena could be detected: 1. discrepancies in the results of flow measurements using different isotopes and 2. a non-uniform distribution of blood flow during experimental ischaemia.

ad 1. Although the CBF measurements took place within 40 minutes and no significant changes took place in arterial blood pressure or $ApCO_2$, the calculated mean flows differed considerably. In all cases the values obtained with ^{85}Krypton were the lowest while with ^{133}Xenon the highest values were calculated. The ratio of disappearance rates of ^{85}Kr/^{133}Xe appeared to be in the same magnitude as the ratio of "diffusitives" of ^{85}Kr/^{133}Xe (0.25). These results indicate that diffusion limitations play an important role. To study these diffusion limitations a series of experiments have been performed using different artificial membranes in a two-compartmental model. The results did, however, not support the hypothesis of diffusion limitation, possibly because of differences between the artificial and biological membranes. This obliged us to initiate a thorough theoretical investigation on this point.

ad 2. The results of the CBF measurements by ^{14}C-antipyrine in small volumes of brain tissue indicate that during experimental ischaemia a non-uniform distribution of blood flow occurs. This point will be confirmed by the quantification of autoradiograms. In the next year these experiments will be prepared for publications or a thesis of Drs. P. A. de Groot, to be published at the end of 1976.

Theme 2: The possible relationship between electrical activity (EEG) and CBF

The main concept of this theme is the demonstration of a relationship between the electrical activity of a particular brain area and its blood flow. It is of interest to estimate the cerebral blood flow – a rather complicated measurement – from the electroencephalogram, because EEG measurements are much more simple and less damaging which is attractive for the clinical situation. The visual cortex of the rabbit provides a good object for this research, since the neuronal activity of this area can be driven by illumination of the retina and CBF can be measured in this area by exact collimation so that rCBF measurements can be performed during different levels of neuronal activity.

The electrical activity of the visual cortex in the conscious artificially ventilated rabbit was altered by means of Intermittent Photic Stimulation (IPS) of the retina. Stimulation frequencies were selected to produce a significant effect on the alpha band parameter of the EEG using a minimal amount of light energy, since the rabbit is nocturnal. The regional cerebral blood flow (rCBF) was measured by means of the intra-arterial injection technique using ^{133}Xenon and electrical activity was simultaneously recorded using bipolar electrodes.

During the resting period there was a significant positive correlation of the EEG and the cortical CBF ($r = 0.96$, $p < 0.001$).

After a critical evaluation of this result, it is assumed not to reflect a causal relationship, since during IPS only the activity in the alpha band was altered significantly ($p < 0.01$), but no concurrent increase in rCBF did occur. This is in contrast to the results of Ingvar *et al.* that seemed to confirm the metabolic theory of autoregulation.

Taking the theta band activity as an indicator for the level of attention of the animal, the hypothesis is tested in a current series of experiments, that rCBF of the visual cortex will increase only if the IPS brings about an attention reaction.

The technical aspects of the experimental set-up were improved again by developing a method of artificial respiration by which the $ApCO_2$ can be changed within a few minutes to every wanted experimental level without changing the respiratory volume. Such a procedure has not been described earlier in the literature.

In order to describe the EEG using few parameters only, two types of computer analysis techniques are used: the frequency-analysis method and the amplitude interval method (in collaboration with the computer department, Ir. J. Smith).

In 1976 the results of this investigation will be published in a thesis by Drs. C. V. de Blécourt.

Theme 3: Effects of radiographic contrast agents on blood flow

This theme is incorporated in FUNGO project no. 13-26-17.

For clinical diagnosis in cases of possible cerebrovascular disorders "four vessel" or "aortic arch" angiography has to be performed. However, the effects of radiographic contrast agents on the blood flow in various organ systems including the brain are not exactly known. A start has been made with the investigation of these effects. This study is undertaken in collaboration with a research group in Utrecht (Dr. G. A. Charbon).

The experiences of theme 1 and theme 2 will join in this item which will be a smaller part of the topic "Cerebral metabolism, CBF, EEG, under various experimental conditions; the effects of vasoactive drugs". This will be initiated in the second half of next year.

MACROMOLECULAR ARCHITECTURE OF CHICK BRAIN SYNAPSES

Participants
Dr. A. B. Oestreicher (biochemist, 100%)
Drs. C. van Leeuwen (biochemist, 100%)
F. Bosman (technician, 100%)
J. W. Maas (technician, 100%)
M. M. Smidt (secretary, 25%)

Theme 1: Isolation and characterization of synaptosomes and synaptic plasma membranes from chick brain

Synapses can be isolated as synaptosomes from brain homogenates after differential and gradient centrifugation. The external, limiting membrane of a synaptosome, the synaptic plasma membrane, contains a specialized part adjoined to the synaptic junction, which is composed of a post-synaptic attachment connected to presynaptic membrane via the cleft material. This junction has been well described morphologically and is in fact the entity through which the communication between nerve cells is occurring.

We intend to identify macromolecules present in the synaptic region that might possess a functional role in neurotransmission or in the establishment of interneural connections during development (see theme 2). Therefore isolation of organelles containing the morphological synapse in highly purified fractions, was a suitable approach. The isolation of synaptosome-enriched fractions has been described in a recent paper (A. B. Oestreicher and C. van Leeuwen, J. Neurochem. *24*, 251–259, 1975).

Initially, isolation of synaptic plasma membranes was carried out according to the method of Cotman and Matthews (1971). In short, this method involves the isolation of synaptosomes from the crude mitochondrial fraction containing mainly mitochondria, synaptosomes and myelin, through a Ficoll gradient in isotonic sucrose followed by osmotic shock treatment and pelleting of the particulate and centrifuging it subsequently on a discontinuous sucrose gradient. Since a persistent portion of mitochondrial contamination was measured in the synaptic plasma membrane fractions, efforts were made to reduce this contamination. For this purpose, the INT method, involving a reaction of the dye INT and succinic dehydrogenase present in mitochondrial membranes, was recommended by Davis and Bloom (1973). Application of their method did not lead to our purpose, but interfered rather with the resolution of synaptic plasma membranes from mitochondrial membranes, presumably by its binding to both membrane types. This phenomenon was neither age nor species dependent, as far as chick and rat brain were concerned. A good yield in synaptic plasma membrane protein was achieved by the rapid floatation-sedimentation method of Jones and Matus (1974). This method is based on differences in buoyant densities of the various types of membranes. Thus, resolution of synaptic plasma membrane from the lighter myelin

fragments and from the heavier mitochondrial membranes might be achieved on centrifugation. The resulting enriched synaptic plasma membrane fraction contained the major amounts of activities of a few putative neuronal plasma membrane marker enzymes: $(Na^+ + K^+)$-activated, ouabain-sensitive ATPase, acetylcholinesterase and 5'-nucleotidase. In addition, markers for various contaminating subcellular elements were decreased in this fraction. Levels of contamination were in agreement with values presented in recent literature on rat brain synaptic plasma membrane fractions.

In near future, morphological and biochemical properties of synaptic plasma membrane-like fractions from chick embryo at different stages of development will be studied.

Theme 2: Specific macromolecules of synapses

Synaptic plasma membranes are known to contain large amounts of $(Na^+ + K^+)$-activated ATPase, an enzyme involved in the transport of sodium and potassium ions across membranes, and of glycoproteins, especially sialoglycoproteins. Recently some speculations have been made on a role of glycoproteins in neural activity and information storage, because of the well-known functions of cell surface glycoproteins in intercellular recognition. It has been demonstrated that a few individual glycoproteins are brain-specific. We assume that specific glycoprotein(s) might be present at the synapses, and thus in isolated synaptic plasma membranes. The $(Na^+ + K^+)$-activated ATPase from the electric eel has been shown to contain a glycoprotein sub-unit.

We are currently investigating the biochemical specificity of the synaptic plasma membrane focus-points, its $(Na^+ + K^+)$-activated ATPase and its glycoprotein. The inhibition by several drugs of the $(Na^+ + K^+)$-activated ATPase activity of the synaptic plasma membrane fraction was studied and compared with their effect on the ATPase of mitochondrial membranes. Preliminary studies on the extraction of glycoproteins from synaptic plasma membranes and classification of the individual glycoproteins will be attempted and their specificity will be assessed by immunological methods.

NEUROPHARMACOLOGY

Participants
Drs. M. van Wijk (biochemist, 100%)
Drs. L. van de Kar (biochemist, 100% until 1.8.1975)
A. M. Lampe-Kraakman (technician, 100% until 1.3.1975)
I. Oberink (technician, 100% until 17.11.1975)
J. van der Velden (secretary, 5%)

In this project serotonin (5-HT) metabolism had a central place.

Theme 1: Mechanism of action of tricyclic antidepressants, especially chronic versus acute effects (M. van Wijk)

This subject was chosen on the ground of the following considerations:

1. 5-HT is probably involved in depression, as is suggested by abnormal metabolite levels of this substance in biological fluids of patients.

2. Tricyclic antidepressants affect the metabolism of this monoamine in the brain, as has been demonstrated in many, mainly acute, experiments on animals.

However, the therapeutic effect of these drugs in patients appears only after at least one week of treatment. Therefore, the relevance of the acute effects observed in animals for the therapeutic effect in man is uncertain. Thus it was decided to compare some aspects of 5-HT metabolism, such as turnover, (re)uptake etc. in animals chronically and acutely injected with several tricyclic antidepressants.

In 1974 it was found impossible to demonstrate a difference in brain 5-HT turnover between rats treated chronically and acutely, respectively, with rather low doses (10 mg/kg) of chloroimipramine, imipramine, desimipramine and protriptyline, although the levels of the drugs in brain and plasma were generally higher in the chronic- than in the acute-treated animals. A decrease in turnover as compared to controls was found after acute administration of chloroimipramine. This reduction, also reported in the literature, appeared to persist after daily injection.

Because the dose used (although still high compared to the clinical doses) might be too low for the rat, the experiment was repeated with a higher dose (25 mg/kg). In this series a difference between chronic and acute effects was indeed found in the case of chloroimipramine: a decrease in turnover was again apparent after a single injection, but disappeared after daily administration. Imipramine caused a decrease acutely which did not persist after chronic treatment; desimipramine and protriptyline did not influence turnover at all. Again the levels of the antidepressants were appreciably higher after chronic than after acute treatment.

At this stage collaboration with Dr. J. Korf (Dept. for Biological Psychiatry, University of Groningen) was started. According to the literature, electrical stimulation of the serotonergic pathways could be used to demonstrate effects on 5-HT metabolism by a dose of 5 mg/kg, much closer to those used in clinical practice. It was thought that this technique might offer a valuable approach to our problem. During a two months stay at the laboratory for Biological Psychiatry, electrical stimulation was practised and applied to the NA pathways, because facilities to study this compound were immediately available. Besides, experiments like quoted for 5-HT had never been reported with respect to NA, while also this neurotransmitter is highly relevant with regard to the action of tricyclic antidepressants. A study was completed, dealing

mainly with the catabolism of noradrenalin (NA) in brain, and using desimipramine as a tool. The results showed the importance of extra-neuronal degradation of the monoamine. After this training the work on 5-HT metabolism was resumed. At present the possibility to adapt a very sensitive microassay for 5-HT, developed by L. van de Kar (see below) for a determination of 5-HIAA is investigated.

Theme 2: The role of 5-HT in the epiphyseo-hypothalamo-hypophyseo-gonadal system (L. van de Kar, see project of Prof. Dr. J. Ariëns Kappers)

The work on an enzymatic radioisotopic microassay of 5-HT was completed and applied to the implantation experiments described in the report of the project of Prof. Kappers. A technique to punch out very small brain particles was finished, to be used for determinations of 5-HT in the hypothalamic nuclei mentioned. Unfortunately, L. van de Kar left for the United States. He will complete this project at the State University of Iowa, USA.

ANGIOARCHITECTONICS OF THE CENTRAL NERVOUS SYSTEM IN SOME LABORATORY MAMMALS

Participants
Dr. A. Miodonski (M.D., biologist, 50%)
B. Przybylski-Zweesaardt (analyst, 35%)
M. Vrielink-Van Dam (analyst, 25%)
S. W. Lust-Bosboom (secretary, 10%)

Theme 1: Angioarchitectonic differences between the gyral and sulcal cortex

The cortex of the higher mammalian brains become convoluted during phylogenesis. It has been shown that the gyral cortex, situated on the top of the gyri, exhibits a different arrangement of nerve fibres and also of nerve cells as compared to the sulcal cortex situated at the bottom of sulci (J. Kreiner). Besides it is known from neuropathological conditions that the sulcal cortex is specially sensitive to anoxia and anoxemia. It is therefore important to investigate whether:

a. the blood vascular pattern is also different in gyral and sulcal cortex, in connection to the anatomical differences mentioned above.

b. The blood vessel can be a causal factor influencing the development of the myelo- and cytoarchitectonics of the cortex. This will be examined first in developmental stages of the rat.

Ad a. In order to solve this problem in the first place we have visualized the cerebral vascular bed in dog, cat, rat and monkey using different injection substances (a mixture of gelatine + india ink; microfil-silicone rubber, micropaque-radioopaque suspension of barium salt). Successful injection of the brain vascular bed depend on factors of which many

are rather out of control, e.g., the health condition of animal (hidden infections), sensitivity to narcotic drugs, diet and hormonal balance, etc. From the animals mentioned were prepared, and will be prepared:

a. histological sections for microscopic examination and,

b. blocks (about 1–1.5 mm thick) which are processed in a special way with chemicals or from which are taken X-ray photo's for visualization of the three-dimensional pattern of cerebral blood vessels, especially in the cortex. The injection procedure on dogs and cats, which needs special operating equipment as well as microröntgenographic examination, has been performed at Prof. Klopper's Experimental Laboratory at the Wilhelmina Gasthuis, Amsterdam. Part of the photographic material is prepared this year for further analysis.

On the basis of our results obtained especially in dog, it appears that there are clear angioarchitectonic differences between gyral and sulcal cortex. This finding needs further confirmation in other mammalian species.

Ad b. We will soon start the preparation of material for a pilot-study on the problem mentioned trying to combine staining of nerve cell processes (Golgi-Cox and Golgi-Kopsch techniques) and the blood vascular pattern in the same brain (see also project Uylings/Veltman). The results could be useful for neuropathologists in explaining why the sulcal cortex is more sensitive to anoxia and anoxemia, and perhaps also to establish the pathological background of some developmental malformations like microgyria or lyssencephalia.

Collaboration is realized with Dr. J. Kus (Department of Descriptive and Topographical Anatomy, Medical Academy, Krakow), who investigates the blood vascular pattern in human embryonic brains.

Theme 2: An angioarchitectonic stereotactic atlas of the rat brain

Such an atlas will be very helpful for neurophysiologists and behaviorists for "damage-free" experimental penetrations into subcortical structures with electrodes. Knowledge of the three-dimensional organization of the vascular bed in the rat brain will allow avoidance of damage of bigger vessels during experiments performed with electrode penetration (chronic implantation, or penetration for making a lesion in some subcortical centres).

For the making of such an atlas it is necessary to prepare injected material from rat brains. Because individual variations exist it is necessary to inject many brains for getting reliable information about co-ordinates of the big and medium basic blood vessels. The material needed is partly ready. Furthermore, several brains are histologically processed for staining nerve fibres and cells after injection of the vascular bed.

Theme 3: Service to other projects

– Preparing histological material (Golgi-Cox, Golgi-Kopsch) for the project of Uylings *et al.* Preparing pilot material for a developmental study on the branching pattern of dendrites for the project of Uylings *et al.* and point b in theme 1 of our project, to be continued in 1976.
– Preparing of histological material for the project of Corner/Baker, to be continued in 1976.
– Histological processing and further analysis with description of the lesioned area in rat brain, for the project of Van der Meché, Bermond and De Bruin. This will be continued in 1976.
– Preparing histological material for the so-called "pineal program" (autonomic innervation of the rat pineal gland, collaboration with Prof. Dr. J. Ariëns Kappers), to be finished in 1976.
– Occasional service to other projects.

Students

J. Cornelisse (analyst training), practising of neurohistological techniques for obtaining the analyst diplome.
P. Wisman (analyst training), practising of neurohistological techniques for obtaining the analyst diplome.

NEUROMORPHOLOGY

Participants
Drs. K. C. Hodde (M.D. 80%)
Drs. P. Kenemans (M.D. 100%)
S. W. Lust-Bosboom (secretary, 15%)
M. A. P. Vrielink-van Dam (technician, 20%)
P. Wisman (technician, 100%)

Thema 1: The structural plan of the lower vertebrate brain stem

The comparative description of a nervous structure aims at developing a conceptual plan by recognizing morphological patterns. Establishing homologies is a focal point in this activity, and historically several criteria for this have been developed, the most important ones being ontogenetic, hodological and topological.

The aim of the present study is to apply these simultaneously onto the same structure, thus establishing optimal descriptive parameters by testing the adequacy and consistency of these criteria. The subject of our study is the brain stem of some primitive and phylogenetically recent fish, viz., *Polypterus*, *Amia*, *Protopterus*, *Scylliorhinus* and *Ginglymostoma*, *Betta* and *Carassius*. This choice was made on the basis of phylogenetic considerations as well as of the availability of data in the literature.

Kenemans, who has been serving military duty during the last 21 months, summarized results in the topological aspects at the Xth International

Congress of Anatomists in Tokio. The standardized method demonstrated allows not only qualitative but quantitative comparison and description as well.

The hodological aspect (K. C. Hodde) has been dealt with by lesioning the spinal cord and tracing the degeneration ascending systems. In part this study was made ready for submittance in co-operation with Dr. Ebbesson (USA).

Some of these systems are much longer than was known previously as is shown by the presence of an uninterrupted spino-thalamic system in *Ginglymostoma* and in *Scylliorhinus*.

This program is to be extended with the Institute's behavioral department. It will, together with the above mentioned topological work, be published as a doctoral thesis in 1976.

Theme 2: The brain of petaurus papuensis ("Sugar Glider")

Together with Dr. Baker (Development of the CNS and Behavior Group), a beginning has been made with the description of the CNS of this marsupial, a relative of the well-known opossum. About ten series, stained according to Nissl, Bodian and combined Fink-Heimer/Nissl are now ready for use. For the most part they were prepared in the Technicum of Histology, during the last six months. Four Fink-Heimer stained series were made of the brains of animals in which one eye was enucleated.

This was done to have a reference-frame in relation to data in the literature on opossum. The brain of *Petaurus* is hitherto virtually unknown. Apart from the visual system, a very general description will be made, and the telencephalic cortex will be subject of a more detailed study.

Theme 3: The central connections of the dorsal roots in frog spinal cord

(See development of the CNS and Behavior, project 6 and 7).

So far, a limited number of tadpoles in different developmental stages and of adult frogs have been ganglionectomized (ggl. dorsal 5, unilaterally) and stained according to the Fink-Heimer technique. This year the pilot-study will be finished by Hodde, who will spend about 30% of his time in 1976 on this subject.

Theme 4: Scanning electron microscopy (SEM) of the postnatal development of the rat visual cortex

Because of the potential of the SEM technique to bridge the gap in between light and electron microscopy we have begun to acquire a working knowledge in this field. First human placenta material was used because of easy availability and an already existing frame of reference in the literature.

Theme 4 was started in November parallel with the work of Uylings and Miodonski and the results so far show the possibility of matching

both ways of approach. In co-operation with the section Electron Microscopy in the Institute the first scanned specimina have been embedded for transmission E.M.

Students/guest workers

P. Wisman (student histology) worked on *Petaurus* and frog material, 1974/75.

J. Cornelisse (student histology) worked on *Petaurus* and frog material, 1974/75.

COMPUTER DEPARTMENT (Bio-informatic aspects in brain research)

Participants
Ir. J. Smith (engineer, 100%)
Ir. K. Kuijpers (engineer, 100%)
M. M. Smidt (secretary, 25%)

In order to automate and quantify the results of various projects complex programs and hardware modules have been developed and used. The computer system we used is an IBM-1130, including the peripherals and a "front-end" Interdata mini-processor.

The Whyte system. In collaboration with the projects "Brain and Behavior" and "Motivational aspects of reproductive behavior" the analysis of sequences of events are investigated. The data from the behavior experiments are recorded in a coded form on seven tracks of an Analog-7 tape cassette. In a daily routine these data are read "off-line" by the computer after decoding. Detailed data analysis is performed hereafter such as: the computation of the frequency, the latency and the total time of the behavior patterns; the transition matrix of the behavior patterns. A further classification of these patterns, using factor analysis and multivariance analysis techniques are in progress.

The radioimmuno assay. In collaboration with Prof. Dr. J. V. Milligan (Kingston, Canada) and the project neuroendocrinology a software package is developed. This program package enables off-line processing of radio-immunological data. In a daily routine the mathematics and diverse plot procedures are executed (for details see project "Neuroendocrinology").

The UV-microscopy. In a daily routine it is now possible to control the rapid tracking and accurate measurements of the fluorescence microscopical sections on-line with the "front-end" Interdata mini-processor. The hardware and software for this purpose were developed last year. This procedure has been developed for the project of Prof. Dr. J. Ariëns Kappers.

The cerebral blood flow analysis. As part of the collaborative research project with the section of experimental neurology, the CBF-curves are plotted and the flow parameters are computed as a routine. In order to state the possible relationship between the electrical activity of the brain

and the CBF, the previously developed EEG-programs are used as a tool. Statistical classification of the EEG-epochs in a few numbers of parameters is therefore important and is now in progress.

The analysis of motility. This theme is performed in collaboration with Drs. W. L. Bakhuis, section of comparative and developmental physiology. The burst patterns of developmental chicken is still being studied. In order to acquire and compare the enormous amount of motility data, each burst is considered to be an event in time. As a first approach the histogram, the tachogram, the mean, the mode and the coefficient of variance of the burst frequency, the burst interval and duration appeared to be representative parameters for the instantaneous intensity of the motility of the animal.

A study is started to use non-parametric statistical test procedures, in order to differentiate between the experiments done under various physiological conditions; between the animals of one experimental group and between the epochs (6–8 hours) of one animal.

Action potentials of neuroendocrine cells and uterine contraction signal analysis. In collaboration with Drs. K. Boer and Prof. Dr. J. V. Milligan an analysis is started of the correlation between the intrauterine pressure changes, represented by an electrical signal, and single unit activity of neuroendocrine cells. Both data are put on Analog tape. As a first approach the data acquisition program is developed, after which specific analysis programs must be developed.

Automatic gaschromatography. In collaboration with the chemical section the project is started to automate the gaschromatograph. The Interdata minicomputer is used for this purpose. The hardware and software development is in progress.

Automatic scanning of dendrites. Topological and metrical investigation on the branching patterns of dendrites is started, using the Interdata minicomputer as a control system. The hardware and software development is initiated in collaboration with Drs. H. B. M. Uylings and H. Overdijk. As part of the increasing needs to connect the experimental set-up on-line to the computer, a multi-task system package is investigated.

Routine statistics. They are performed for practically all projects.

Computer time. The mean "computer time" (as read on the computer clock) per week is 52.25 hours. This indicates how much the apparature has to be used during evenings and week-ends by the increasing interest of the various groups in these techniques.

In the progress report 1974 the following types of quantitative EEG analysis were reported:

a. the spectral analysis using the first fourier method
b. the aperiodic extreme interval method, and
c. the pattern recognition method.

The results of the quantitative EEG-analysis of the developmental chicken were assembled and closely examined in collaboration with G. Runhaar. In normal ontogeny the quantitative analysis of the developmental EEG from stage 42 to 45 has indicated that an increase in power (i.e. amplitude) takes place, especially in the δ-band. This can be seen in the spectral analysis as well as in the amplitude-analysis. During these periods functional cerebral development in the chick embryo normally involves the appearance of large amplitude and fairly stereotyped potentials at about stage 42/43. Probably these large amplitude slow wave complexes, appearing at variable intervals and superimposed upon the higher frequency background electrical activity, reflect the state of reverberations in underlying neuronal nets.

In order to classify more directly the question of EEG organization in time, an EEG-pattern recognition algorithm was developed, for computing the spontaneous, bioelectrical activity with fairly stereotyped potentials.

Our model of the stereotyped patterns in the EEG is characterized by a short amplitude peak of 15–100 μV having an interval of 150–250 msec, and a relatively long afterpotential of 10–40 μV having an interval of 200–250 msec between stage 42 and + 2. The average number of stereotyped patterns per minute for a false alarm probability of 0.1 varied from 40 to 55 for stage 42 to 45. While after birth a frequency shift occurs to the higher frequencies: alpha-band. All three types of analysis methods proved to be an objective method for quantifying the EEG for the developmental chicken.

Students/guest workers

E. Smienk (HTS-electronics), The digital input for the fluorescence microscope.
– The ULI-Interface.

L. J. Bouma (HTS-electronics), The interface of the Ampex digital tape-unit.

P. Kolber (HTS-electronics), Investigation of microcomputers.

J. C. Groot (HTS-electronics), Software development in order to automatize the gaschromatograph.

H. Steigstra (HTS-electronics), Hardware development for gaschromato graph.

G. Runhaar (student biology), Development of the EEG in the chicken.

ANIMAL QUARTERS

F. Harkema, animal care taker 100%
R. Hofer, animal care taker 100%
Dr. A. B. Oestreicher, coordinator

The number of research animals in the animal quarters consists continuously of 30 rabbits (New Zealand or Bastards), 800–1000 rats (Wistars, S_3, Brattleborros), 50 frogs (Rana pipiens, esculenta, temporia) and 250 chicken eggs. 30 Chicken hatch each week. In addition there are about 400 fish in our main building (Carassius auratus, Betta splendens) divided over 60 aquaria.

In order to improve the existing provisions and to enable a necessary extension of the number of research animals (in 1975 more than 50%) the animal accommodations underwent a complete reorganisation from June 1974 until the end of 1975.

A washing machine for animal cages and movable racks was installed. As a result of the installation of movable racks for animal cages the capacity was three times enlarged. The breeding machine for chicken eggs was replaced by a new one. A new chicken unit with automatic food and water supply was installed.

In order to make cleaning and desinfecting of the animal quarters possible, the walls were covered with eternite plates. In addition the animal quarters were painted.

The reorganization and new provisions directed to an optimalization animal care, resulted already in a clear fall in the appearance of diseases in both, rats and rabbits.

MECHANICAL WORKSHOP

Participants
A. W. Kamstra (technician, 100%)
E. Moes (technician, 100%)

An enumeration of some large projects developed in this department is given below. Electronical aspects of the projects were developed in the electronical department.

Self-stimulation boxes for rats (N. E. van de Poll)

Plexiglass boxes (4) with lever and microswitch for electrical self-stimulation were built in which the current is triggered by the rat when a specific side of the box is chosen. All boxes are equiped with a rotating swivel, and an electrical contact.

Boxes for observation of sexual behavior in the rat (N. E. van de Poll)

A unit of 12 boxes was built for the observation of sexual behavior of rats. All boxes are provided with a device to enter the receptive females automatically at the command of the observer.

Scanner-system (J. de Bruin, B. Bermond and N. E. van de Poll)

Keyboards and mechanical parts of the scanner and interfaces of the "White Scanner System" and Grason-Stadler programming units were built in collaboration with the electronical workshop (for details see Electronical Workshop).

Rat stereotact (A. R. Smith)

Several adaptations on a stereotact that has been built earlier in our workshop were made, in order to get full control on the position of the micromanipulator and the rat's head in the headholder.

Fish tanks of plexiglass (J. P. C. de Bruin)

Large tanks were built in order to store a number of fish in visual isolation. Smaller ones were developed to measure levels of sexual behavior in male fish. Observation cages to be used in an experimental set-up to measure levels of aggressive behavior in rodents.

Microscope stage (H. Uylings)

The instrumental error of two GFL microscopes is determined in 3-dimensional point measurements. Since the normal slide-holder and microscope stage is not appropriate enough for accurate point measurements and for quantitative studies, a new microscope stage has been constructed. One of these two GFL microscopes will be used for a semi-automatic system for measurement of dendrite branching.

Adjustable preparation table (M. A. Corner)

A preparation table, suitable for electrophysiological experiments on the spinal ganglion of the frog, was developed, while a self-made motorized micromanipulator has been revised.

Egg holder (W. L. Bakhuis)

An egg holder was made such as to permit equal mechanical forces applied to the intact egg-shell by the embryo, enclosed in it, to exert equal forces on a movement transducer.

Holder for opened egg (W. L. Bakhuis)

A modification of the intact egg holder was made by equiping it with a head holder. It is used in experiments in which movements are registered of foetuses with the head pulled out of the egg.

Movement transducer calibration

A small motor was fit in the above mentioned holders to calibrate the movement transducers.

Frog skin holder (J. du Pont)

We have developed a set-up for the isolated frog skin. This device makes it possible to record from all possible skin fibres and to regulate the temperature. Besides a precise registration of the stimulus co-ordinator it made receptive field plots possible.

Polygraph motor (K. Boer)

A new motor was built in a Schwarzer polygraph in order to write out in an appropriate way intra-uterine pressure changes concurrently with single unit recordings from the brain. This motor allows a much wider speed range, both slower and faster, as the original one.

Homogenizers (G. J. Boer)

Micro-pestle homogenizers were made for homogenization of mg quantities of tissue in 25 to 50 μl.

ELECTRONICAL WORKSHOP

Participant
J. Overdijk (technician, 100%)

An enumeration of some larger projects developed in this department is given in the following section. Mechanical aspects of the systems were developed and built by the mechanical workshop.

1. *White's behavioral registration system* (Key-board, Scanner and Decoder) built for J. de Bruin, B. Bermond and N. van de Poll.

The White system was developed and modified, based on literature data and on personal information provided by Dr. R. A. C. White (Institute of Animal Behaviour, Rutgers University, Newark, USA), in collaboration with the Computer section. The system consists of a keyboard with 20 keys and a built-in scanner which scans the position of the 20 keys 20 times per second. This information is encoded and stored on a magnetic tape (Analog 7).

Transcription on digital tape and further analysis takes place in the Computer section. The Electronical Workshop developed and built in a decoder while the Computer section provided the necessary software for transcription and storing the information in a reduced form on digital tape. The tapes are decoded at 8 times the original speed. The first keyboard (with scanner and encoder) and the decoder were finished in 1974, while two additional keyboards were made in 1975. They are provided with an additional feature which enables an extra input of 20 data, e.g., derived from instrumental conditioning or another keyboard.

2. *Interface Scanner-System and Grason-Stadler 1200 programming system* (N. van de Poll)

An interface between the scanner and the Grason-Stadler programming units enables a reliable registration and computer analysis of the learning schedules and behavioral data of the rat in the skinnerbox. Moreover, behavioral observations can be registered and analysed together at the same time, by means of a keyboard.

3. *Artifact free body temperature controlled heating pad* (K. Boer)

Developed and built a heating pad for an electrophysiological recording set-up. The quintessence is that the body temperature of a small animal regulates the current flow through the heating coil without using relay-switches in order to avoid any artifact induction. An animal can be brought and kept at a certain temperature within a limit of 0.2° C. Furthermore, the measures are compatible with space available under a small animal in a conventional David Kopf stereotact.

4. *Lecture synchronizer* (D. F. Swaab)

Developed: a lecture synchronizer in order to allow 6 synchrone lectures during the Xth Acta Endocrinological meeting in Amsterdam. The costs are reimbursed by the congress-committee.

In development are:

1. *Section of Ethophysiology* (A. P. van der Meché) *Central steering-unit for 6 shuttle-boxes.*

In 1975 a pilot model has been developed, built with integrated circuits, to replace the presently used central steering-unit.

2. *Semi-automizing of 3-dimensional dendrite measure system* (H. Uylings)

The development is started of the electronic part of a semi-automatic system for measuring 3-dimensional co-ordinates of joints in microscopic slides (for measuring branching patterns of dendrites see project H. Uylings). A joy-stick generator/drive has been developed already, which is computer time saving since it can act without using the computer. Furthermore, specific 16 bits up-down counters, buffer- and shift registers of the measurement-signals have been developed. In 1976 two demodulators for connecting the system with the Interdata Computer will be built.

3. *Device that starts registration or other apparatus as soon as hatching activity commences* (W. L. Bakhuis)

In studies implicating registration of phenomena that have a short duration, while the moment of start is inpredictable, much time is lost waiting for the phenomenon to appear. Hatching (climax) activity is such a phenomenon. To solve this problem an apparatus has been developed that scans the transduced output of embryonic motility every 5 minutes. The apparatus discriminates intervals between 6 and 20 seconds from other intervals between motility bursts and only the former are counted as activity. As soon as the activity surpasses a pre-adjusted level, the apparatus starts another apparatus, i.e., an analogue tape recorder and/or video-recorder.

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HUBRECHT LABORATORY

INTERNATIONAL EMBRYOLOGYCAL INSTITUTE - UTRECHT

Progress Report 1975

History and objectives of the Institute

The Hubrecht Laboratory was founded in 1916 in memory of the Utrecht zoologist and embryologist Prof. A. A. W. Hubrecht. It is a semi-governmental institution operating under the supervision of the Royal Netherlands Academy of Arts and Sciences.

The objective of the Laboratory is to function as an *international research and service centre for developmental biology*. To ensure a multidisciplinary approach to the many problems of development eight disciplines are being practised, each applying a variety of experimental procedures (see under Scientific Staff below).

The Laboratory aims at stimulating international co-operation and understanding by, among other things, organising International Research Groups in Developmental Biology at more or less regular intervals, and by the biennial publication of an international directory of investigators active in developmental biology (General Embryological Information Service).

The Laboratory houses the Central Embryological Library (collection of reprints covering the field of developmental biology) and the Central Embryological Collection (microscope slides and material preserved in alcohol).

Individual guest workers are welcome at the Laboratory. Partial financial support is available in special cases only.

Management and Scientific Staff

P. D. Nieuwkoop, Ph. D. – Director, Prof. of Experimental Embryology, University of Utrecht
J. Faber, Ph. D. – Deputy Director
Elizabeth A. Berends – Laboratory Manager

B. Z. Salomé, M.Sc. – Chief Librarian
Romee Verhoeff-de Fremery, M.Sc. – Supervisor of animal care

J. G. Bluemink, Ph. D. – Ultrastructural research
Elze C. Boterenbrood, Ph. D. – Experimental morphology; Curator of the Central Embryological Collection
A. J. Durston, Ph. D. – Developmental physiology
K. Hara, Ph. D. – Experimental morphology
S. W. de Laat, Ph. D. – Biophysics
Kirstie A. Lawson, Ph. D. – Tissue and organ culture
W. J. Ouweneel, Ph. D. – Developmental genetics
P. T. M. van der Saag, Ph. D. – Biochemistry
Geertje A. Ubbels, Ph. D. – Histo- and cytochemistry

Visiting Scientists – 1975

R. J. Ransom, Ph. D. (Edinburgh, U.K.)
K. Rzehak, Ph. D. (Kraków, Poland)
H. H. Seydewitz, Ph. D. (Saarbrücken, B.R.D.).

Temporary Research Assistant – 1975

R. T. M. Hengst, M.Sc.

Semi-Scientific Staff (Technicians) – 1975

A. R. J. Bleumink
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Cornelie Koning
W. A. M. van Maurik
Jannie G. Stroop-Wijchman
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Willeke M. Vonk

Graduate Students (University of Utrecht) – 1975

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A. S. Koster, B.Sc.
W. Lammers, B.Sc.
K. Weyer, B.Sc.

(For Dutch workers B.Sc. and M.Sc. are used as the approximate equivalents of the Dutch degrees of Biol. Cand. and Biol. Drs.)

INTRODUCTION

A fifth major area of research, morphogenesis in the cellular slime moulds, has been added to the research programme of the Laboratory. The five major areas are represented by sections I–V below, following the order from the broadly organismal and organ levels to the more strictly cellular and molecular levels.

I. Embryogenesis in amphibians: origin and establishment of embryonic axes; analysis of morphogenetic movements

This section reports on work at the supracellular and cellular levels involving amphibian embryos. The two major areas of interest are: (1) the analysis of regional differences in the fertilised egg and the cleavage and blastula stages, in connection with the establishment of the future dorso-ventral and antero-posterior axes of the embryo and mesoderm induction; (2) cellular and supracellular mechanisms involved in the morphogenesis of the gastrula and neurula.

Work on amphibian embryos at the subcellular and molecular levels is reported in section V.

A. Experimental studies on cleavage stages

1. *Dissociation and re-association of early embryos (Xenopus laevis)* (K. Hara)

In order to investigate the state of dorso-ventral polarisation in both animal and vegetative blastomeres and its possible changes during development, experiments are being planned to recombine blastomeres isolated from 32-cell and possibly younger embryos. The following preliminary experiments were performed.

Fertilised eggs were decapsulated and placed in 1/15 M phosphate buffer solution (pH 7.8) with the vitelline membrane intact. Cleavages proceeded at the normal rate but the blastomeres lost their normal contact, exposing part of the newly formed cell membranes to the outside. When the vitelline membrane was removed at this time the embryo fell apart into single blastomeres. However, when the embryos within their intact vitelline membranes were returned to Holtfreter solution or sterile tap water after the 3rd, 4th or 5th cleavage, almost all of them reformed a continuous surface layer and developed into normal larvae.

In contrast, such re-association after the 6th or later cleavages resulted in progressively more abnormal embryos. Both direct observation and cinematographic analysis revealed that several of the dissociated animal superficial cells disappeared into the interior of the embryo, leaving scattered gaps in the surface layer upon re-association of the embryo. Apparently the reconstitution of a complete surface layer is essential for further normal development.

B. Mesoderm induction in the blastula

1. *Embryonic axis formation in recombinates of reaggregated endoderm and intact animal cap material from blastulae (Ambystoma mexicanum)* (P. D. Nieuwkoop)

This year there was time only for a preliminary analysis of the material of the series of experiments mentioned in the previous report (sect. I.B.1). The results are as follows. (1) Axis formation occurred in nearly all recombinates as a result of mesoderm induction by the reaggregated dorsal endoderm. (2) Most of the embryonic axes were single, but there were also recombinates which formed double or triple axes. (3) Nearly all axes showed local and partial reduplication of the notochordal rod. This is a strong indication of secondary fusion of mesodermal masses before and during the segregation of the notochordal anlage.

These observations support the idea that mesoderm induction may start at several points along the periphery of such recombinates (in the central portion a new blastocoel develops, which precludes inductive interactions, as in the normal embryo). Depending upon the spatial

distribution and size of the initial mesodermal inductions, these may then fuse into a single axial system or into two or more separate axes.

2. *Pattern formation in the dorsal marginal zone of the blastula and the archenteron roof of the gastrula (Triturus alpestris)* (K. Weyer – an experimental-morphological analysis in conjunction with a computer simulation of mesoderm induction; in collaboration with the Department of Theoretical Biology, State University of Utrecht)

This is a continuation of work started last year (see report for 1974, sect. I.B.3). The requirements were established for a computer simulation of the extension into the animal, ectodermal cap of the blastula of the mesoderm-inducing action emanating from the vegetative yolk mass. It was concluded that more experimental data should be collected in order to make such a simulation feasible.

The second part of the study concerned the interactions between the neuro-ectoderm and the mesoderm of the gastrula. Recombinates were made of invaginated archenteron roof material with either competent neuro-ectoderm of the gastrula or non-competent ectoderm of the neurula (controls). The results suggest that during gastrulation the neural anlage exerts a notochord-enhancing influence upon the underlying archenteron roof.

C. The origin of bilateral symmetry in amphibian eggs

As mentioned in previous reports, a central region of "clear cytoplasm" is present shortly after fertilisation in eggs of two anuran species, *Discoglossus pictus* (see sect. VIII, ref. 6) and *Xenopus laevis* (see ref. 10). Prior to first cleavage this cytoplasm is progressively displaced towards the future dorsal side of the egg and is then called the "dorsal cytoplasm". This is a constant phenomenon and is the first internal sign of bilateral symmetry in the egg. It may be related to the final bilaterality of the gastrula, which is foreshadowed by the establishment of dorso-ventral polarity in the endodermal yolk mass of the blastula (see previous reports, sections on mesoderm induction). However, a direct connection between the displacement of the clear cytoplasm and the polarisation of the endoderm has yet to be demonstrated (see also section I.A.1 above).

1. *The possible factors involved in cytoplasmic movements in the fertilised egg (Xenopus laevis)* (G. A. Ubbels, K. Rzehak)

Dorsal displacement of the clear cytoplasm is never observed in eggs that are activated but not fertilised. This suggests that it is evoked by the entrance of the sperm. However, in *Xenopus* eggs activated by exposure to thioglycolate or tap water characteristic shifts of surface pigment do take place and sometimes even lead to the formation of a pseudo-grey

crescent. This suggests that the shifts of surface pigment involved in grey crescent formation and the internal cytoplasmic displacements are not necessarily causally related. This conclusion is supported by the fact that in fertilised *Xenopus* eggs grey crescent formation takes place after the dorsal displacement of the clear cytoplasm is completed, in contrast to the situation in *Discoglossus*.

Our hypothesis is that the entrance of the sperm evokes and/or activates a kinetic system that is responsible, first for ooplasmic segregation and then for the dorsal displacement of the clear cytoplasm. This hypothesis is being tested in two ways: (1) a light-microscopic study is being made of the relations between the spermaster(s) and the clear cytoplasm in artificially fertilised monospermic and dispermic eggs, and of the relations between the female pronucleus monaster, the cytasters, and the clear cytoplasm in spontaneously activated eggs; (2) newly laid eggs are exposed to or injected with substances which are known specifically to inhibit the formation and/or functioning of microtubules (colchicine, colcemid). (Preliminary results show that it is possible reversibly to inhibit the first cleavage division by colchicine treatment).

Another approach is based on the fact that biogenic amines are known to play a part in cytoplasmic movements in various animal cell types (cf. previous report, sect. I.C.1). Uncleaved eggs are being treated at various times after oviposition with relatively high concentrations of substances which specifically inhibit the synthesis of biogenic amines (such as β-phenylethylamine) or are assumed to interfere with their activity (such as chlorpromazine, cyproheptadine, and LiCl).

For the cytochemical evaluation of the results of such experiments application of the Falck reaction for the localisation of biogenic amines is necessary. The freeze-drying procedures required for this reaction have now been worked out for both *Xenopus* and *Discoglossus*. In *Xenopus* eggs at 50 mins. after oviposition (end of dorsal displacement) the dorsal cytoplasm is only very weakly positive, but earlier stages are now being investigated and more sensitive procedures will be tested.

All size classes of yolk granules show autofluorescence with this test, with excitation peaks at 335, 375 and possibly 410 nm and an emission peak at 445 nm (monochromator at 380 nm). More data must be obtained before a reliable interpretation will be possible.

2. *The morphogenetic role of the "clear cytoplasm" in the fertilised egg (Xenopus laevis, Discoglossus pictus)* (R. T. M. Hengst)

The experiments on *Discoglossus* involving transfer of dorsal cytoplasm from one egg to the ventral side of another egg (see previous report, sect. I.C.2) were extended. Twenty nl of cytoplasm was transferred. Of the donor embryos 38% yielded larvae. Of the host embryos, 44% were arrested at gastrulation and several showed exogastrulation. Of the

embryos surviving till larval stages, 45% of the donors and 57% of the hosts showed axial malformations.

As a control for dorsal cytoplasm removal, *Xenopus* eggs were fixed immediately upon cytoplasm extraction and processed for histology. In 91% of the eggs the dorsal cytoplasm had indeed been removed; the pronuclei or zygote nucleus had always remained behind. Cytological disturbances were frequently encountered, which were apparently due to the experimental procedure: (1) internal pigment aggregates located far centrally from the animal pole area, and (2) one or more centrally located "empty" cytoplasmic areas. Whether the latter had contained substances dissolved out during processing for histology will be tested by appropriate cytochemical methods.

Preliminary experiments in which dorsal cytoplasm extracted from a *Xenopus* egg was injected below the ventral cortex of the same egg yielded the following results: (1) experiment performed at 45–60 mins. post oviposition (period of grey crescent formation and syngamy): mainly developmental arrest before or during first cleavage; (2) 60–70 mins. p.o. (period of nuclear division): larvae with axial malformations; (3) 70 mins. p.o. till first cleavage (preparatory period for cleavage): either arrest during cleavage or normal development. These experiments are being extended.

As mentioned in the previous report (sect. I.C.2), in dorsal cytoplasm transfers from one *Xenopus* egg to the ventral side of another egg the percentage of axial malformations in surviving larvae is lower in the hosts than in the donors. For the interpretation of this difference it is necessary to know the exact relation between the site of wounding and the location of the later dorsal blastopore. To this end a method was developed to stain the egg at the site of wounding. A micropipette of 50 μm tip diameter, plugged with porous material just below the tip and filled with nile-blue sulphate solution, is placed on the egg surface next to the transplantation micropipette for 2 mins., the stain solution being forced out by slight pressure. In this manner a long-lasting colour mark is obtained.

D. The control of cell behaviour during morphogenetic movements

1. *Time lapse cinematography of gastrulation movements (Ambystoma mexicanum)* (A. J. Durston)

The investigations described in section I.D.1 of the previous report were continued. Whole gastrulae (stages 10½–11) were also opened so that films could be made directly of the cells of the invaginating mesodermal mantle. The films made so far reveal that the usual culture media interfere with normal cell behaviour during gastrulation. An appropriate medium is being sought.

2. *The biogenic amines as possible morphogens for gastrulation and neurulation movements (Xenopus laevis)* (A. J. Durston)

This is a continuation of the work reported in section I.D.2 of the previous report. There are claims in the literature that biochemicals affecting biogenic amine synthesis, and a biogenic amine affect *Xenopus* gastrulation and neurulation movements. Pregastrula embryos were exposed to maximal sublethal concentrations of more than 20 biochemicals known to affect biogenic amine synthesis or action. These were: biogenic amines, precursors, and known inhibitors of their action, secretion, synthesis or breakdown. All of the agents were added externally to decapsulated embryos in Steinberg solution. Some were also injected into the blastocoel of the st. 9 embryo (4 substances) or into the cytoplasm of st. 2 or 3 embryos (5 substances). The test embryos were scored for delays or abnormalities in gastrulation and neurulation.

The reported effects of the two synthesis inhibitors diethyl-dithiocarbamate and 1.10 phenanthroline (Gustafson, 1971, 1973) were confirmed. Effects of phenylethylamine (Stanisstreet, 1974) were less striking. None of the other agents tested had a very considerable effect on gastrulation or neurulation. Injection did not enhance the effects of ineffective inhibitors.

The effects of the two clearly effective synthesis inhibitors could not be reversed by biogenic amines or appropriate precursors. It should be noted that these two agents are not *specific* inhibitors of biogenic amine synthesis: they are copper chelators and should affect a number of enzyme-catalysed reactions. These data do not support the notion that biogenic amines have a role in *Xenopus* gastrulation or neurulation.

Gastrulae (st. 10–12) and neurulae (st. 13–17) were also freeze-dried and exposed to formaldehyde vapour (which is reported to react with biogenic amines to produce identifiable fluorophors; Falck, 1962). The whole embryos were then sectioned and examined for specific fluorescence. Treated embryos were found to be more fluorescent than controls, in which the yolk shows considerable autofluorescence. The induced fluorescence observed was also localised in yolk platelets. Spectral analysis showed that it has the excitation and emission maxima reported for two tryptophan dipeptides (Edvinsson *et al.*, 1971, Hakanson *et al.*, 1971). These data give no reason to suspect that *Xenopus* gastrulae or neurulae contain high concentrations of biogenic amines.

II. Pattern formation in insects

Insect imaginal discs are eminently suited for the study of pattern formation in multicellular systems. Both genetic and experimental-morphological methods are being used for the analysis of pattern formation.

1. *Morphogenetic studies on imaginal discs with genetic techniques (Drosphila melanogaster)*

Major problems in the study of the normal morphogenesis of imaginal discs are the following: the number of initial imaginal cells in the anlage of the disc in question in the blastoderm; the growth and morphogenesis of the disc itself; and the determinative compartmentalisation of the disc. Clonal analysis by means of X-ray-induced somatic crossing-over (SCO) is the best technique available for the study of these problems. Another technique is the use of position-effect variegation (PEV).

1a. The haltere disc (W. J. Ouweneel, W. Lammers, W. M. Vonk)

In continuation of work described in the previous report (sect. II.3a) morphogenesis of the haltere disc was studied by means of clonal analysis through SCO. The best results have so far been obtained with the help of the genetic marker *mwh (multiple wing hairs)*, which affects the number and orientation of trichomes. Embryos and larvae were irradiated at four different ages. Only 3% of the 5400 halteres examined exhibited *mwh* clones; their frequency increases with the age of irradiation. The sizes, shapes and positions of the clones are being analysed and have supplied preliminary information on mitotic rate and orientation during development and on the division into the major compartments of the haltere disc (anterior *vs* posterior, dorsal *vs* ventral, haltere *vs* metathorax, and capitellum *vs* pedicel *vs* scabellum). The use of *Minute* mutants has been developed further to improve the method.

1b. The eye disc (R. J. Ransom, W. J. Ouweneel)

Clones were induced in eyes of various *Drosophila* stocks by PEV or SCO. First, a microscopic study of thin sections (1 μm) showed that at the edge of a clone the cells of individual ommatidia sometimes lie only partly inside the clone boundary (this holds for either type of clone). In addition, PEV clones often contain "pink" areas, which are apparently due to sparseness of pigment. Perhaps PEV and SCO produce clones in different ways.

Secondly, the orientations of SCO-induced clones on the head cuticle (using the markers *yellow*, *white*, and *singed*^{2}) were compared with those predicted by a computer model of eye disc development (see previous report, sect. II.3b). The cell division patterns inferred from the observed clone orientations satisfactorily matched those predicted by the model. It was also shown that, although posterior clones in the eye proper (which arises from the central part of the eye disc) are smaller than anterior ones, this does not hold for clones in the surrounding head cuticle. These results are being considered within the framework of a theoretical model of cell interactions during development (R.J.R.).

2. *Duplication in imaginal discs by early X-irradiation* (W. J. Ouweneel, W. M. Vonk)

So far duplication of haltere structures has been encountered in two different situations: (a) after *in vivo* culturing of posterior haltere-disc fragments in adult hosts and subsequent back-transplantation into mature larvae (see previous report, ref. 9), and (b) in certain mutants such as *costal*, which produces similar duplications, possibly as a result of cell death in the disc (at present under investigation) followed by excessive cell growth into the degenerated region (see previous report, sect. II.2).

A third approach has now been followed. Larvae of 20 hrs. were X-irradiated with high dosages (1000 r and more). This often seems to kill part of some discs, leading to subsequent compensatory cell proliferation. Haltere duplications were observed in 4.5% of the irradiated animals (as well as leg duplications in fewer cases). These are very similar to, but often more extreme than haltere duplications in *costal* flies; the orientation of the duplication process seems to follow the same rules as in cultured disc fragments.

3. *Genetic interactions between homoeotic mutations during development* (W. J. Ouweneel, W. M. Vonk)

The recent strong emphasis on compartmentalisation in imaginal discs, a process probably controlled by homoeotic mutants, has revived interest in homoeotic "transformation series" in which a mutation changing structure A into B is combined with a mutation changing any structure B into C. Newer studies have revealed that *tetraltera* changes the wing into a mesothorax (which is therefore present in duplicate); combination with *ophthalmoptera* (which changes eye area into wing) produced mesothoracic structures in the eye. On the other hand, combinations of *opht* with *Contrabithorax* (which changes wing into haltere) did not so far produce haltere tissue in the eye.

III. Epithelial-mesenchymal interactions in organogenesis

The pattern in which a branching epithelium develops, such as that of the salivary gland, lung or kidney, is characteristic for each organ and can be described in terms of points of branching and growth of the epithelium. It has long been known from organ culture experiments that the investing mesenchyme is essential for both growth and morphogenesis of these epithelia. Recombinates of epithelium and mesenchyme are being used to investigate branching morphogenesis *in vitro*.

1. *Electron microscopy of early stages of branching morphogenesis in the lung (Mus musculus)* (J. G. Bluemink, W. A. M. van Maurik, K. A. Lawson)

This work is a continuation of that described in the previous report (sect. III.3). Its purpose was to examine the epithelial-mesenchymal

interface in the lungs of mouse embryos *in vivo*. The study is finished and the results can be summarised as follows.

The epithelial-mesenchymal interface in 11-, 12- and 13-day lung primordia was analysed at the ultrastructural level with emphasis on the occurrence of cell contacts and extracellular matrix organisation. In 11-day lung (primary bronchus) the basal lamina is intact, but occasionally cell surface extensions establish intimate cell contacts between epithelium and mesenchyme in the distal region of the primordium. In 12- and 13-day lung the basal lamina is partly absent along distal regions of outgrowth and sometimes the domains of epithelium and mesenchyme are difficult to define. Intimate cell contacts between epithelial and mesenchyme cells (50–90 Å separation) are regularly seen.

Collagen fibres (130 Å thick) having a 30 Å striation pattern are found mainly along quiescent regions. In general extracellular matrix material is very scanty along distal regions of outgrowth. Aggregates of electron-dense vacuoles are found almost exclusively in the basal cytoplasm of epithelial cells and are connected with the RER. They show morphological characteristics reminiscent of collagen-secreting vacuoles.

It is suggested that transient intimate cell contacts between epithelium and mesenchyme may be instrumental in the initiation of bifurcation of epithelial buds. The results have been submitted for publication.

2. *Transfilter interaction in lung morphogenesis (Mus musculus)* (A. S. Koster, K. A. Lawson)

Taderera (1967) carried out *in vitro* experiments in which Millipore filters were interposed between 12- and 13-day mouse lung epithelium and mesenchyme. Certain conclusions were drawn from the results, notably that no cell-to-cell contacts are required for epithelial branching. The reasons for re-investigating this problem are twofold: (1) cell contacts do occur between epithelium and mesenchyme of the developing lung *in vivo* and *in vitro* (see 1 above and previous report, sect. III.3) and (2) newer studies using Millipore and Nuclepore filters in another system (Wartiovaara, Saxén, and others, 1972–75) have led to a reconsideration of the results of previous transfilter experiments.

A study was initiated to establish a suitable culture system for transfilter experiments using Millipore and Nuclepore filters. Differences in epithelial morphogenesis of intact lung rudiments were found between different culture systems, but they seem to be explainable in terms of the amount of viable mesenchyme present next to the epithelium. This amount may in its turn depend on the embryonic age of the lung rudiment as well as on the surface structure of the filter used and the nature of the medium.

IV. Morphogenesis in the cellular slime moulds

The control of cell behaviour during the aggregation stage of development

in *Dictyostelium discoideum* (Dd) is well understood. The aggregation control system of Dd is, at this time, the only morphogenetic field which is understood at the cellular level. The following lines of investigation into *later morphogenesis and pattern formation* in this organism have now been started.

1. *Analysis of the formal features of pattern formation in the slug (pseudoplasmodium) (Dictyostelium discoideum)* (A. J. Durston)

The Dd slug is a dynamical pattern-forming system. It has a regulative axial pattern of differentiation and various axially polarised properties which regulate following injury. The anterior boundary region of the slug (tip) has a role in controlling this regulative behaviour: secondary tips grafted into slugs set up secondary axes of differentiation. A number of experimental approaches have been started, which are aimed at determining quantitatively some of the parameters of pattern formation in the slug and its control by the tip.

2. *The role of the aggregation control system in later development (Dictyostelium discoideum)* (A. J. Durston, K. Weyer)

Several lines of evidence suggest that elements of the Dd aggregation control system are important for later morphogenesis. We are pursuing some of these. Two approaches are: (1) development of methods for tracking individual cells in later structures, to get an idea of how cell movement is controlled in later stages; (2) detailed analysis of patterns of cyclic AMP secretion by later structures, to determine which, if any, of the modes of cyclic AMP secretion known to control cell movements in aggregation persist in later stages (with K. W.).

3. *Genetics and morphogenesis (Dictyostelium discoideum)* (A. J. Durston, K. Weyer)

We are making and collecting morphogenetic mutants of Dd with defects in later morphogenetic processes. These will be used to assist in elucidating causal relationships between cell properties of interest and later morphogenesis and pattern formation.

V. Regulation of the cell cycle: role in development and differentiation

This is a collaborative project of the research units of biochemistry, biophysics, and ultrastructural research. The research is being focussed primarily on the plasma membrane-cytoplasmic interactions which possibly play a role in the regulation of the cell cycle and thereby in the initiation of cellular differentiation. A simple model system (neuroblastoma cells) has been introduced.

1. *Plasma membrane-cytoplasmic interactions during the cell cycle and differentiation of murine neuroblastoma cells* (S. W. de Laat, P. T. van der Saag, J. G. Bluemink, C. M. A. W. Festen)

The investigation uses C 1300 murine neuroblastoma cells (clone A2). For a multidisciplinary research project such as the present one a number of technical requirements must be met. These will be discussed first.

(i) *Mitotic synchronisation* of the cells must be possible, preferably by a method that does not interfere with cell metabolism. Shaking off dividing cells from a monolayer is an example. This method was worked out for neuroblastoma cells. The degree of synchronisation was determined by means of ^{3}H-thymidine incorporation and autoradiography, and time-lapse photography. For 80% of the cells the first cell cycle after shake-off takes 7–9 hrs., the same duration as that determined in time-lapse pictures of asynchronous cultures. The S-phase occupies 5–6 hrs. The very short cycle duration ensures a good yield of synchronised cells.

(ii) *Isolation of plasma membranes* must be possible to study their chemical composition and physical properties, as well as the activities of membrane-bound enzymes. Three different methods were worked out for neuroblastoma cells: (1) isolation upon stabilisation with $ZnCl_2$, (2) isolation from latex particles taken up by the cells, and (3) isolation via infection of the cells with Semliki Forest virus (the membrane of the reproduced virus particles is identical in lipid composition with the plasma membrane —this method was developed in collaboration with Dr. B. A. M. van der Zeijst, Lab. of Veterinary Virology, University of Utrecht). The isolated membrane fractions were shown to be free of cytoplasmic contamination by electron microscopy. The study of the activities of membrane-bound enzymes has started.

(iii) *Electrophysiological measurements* in cells of such small size (10–20 μm) pose special problems. These were overcome by constructing a device by which it is possible to move micro-electrodes into the cell in steps of 1 μm.

(iv) A special method is required for *freeze-fracture EM studies* of the plasma membrane. A new technique was developed for breaking cells growing in a monolayer on glass without removing them from the substrate (L. G. J. Tertoolen, in collaboration with Dr. P. H. Ververgaert and Dr. A. J. Verkleij, Biological Ultrastructure Research Unit, University of Utrecht).

A beginning was made with the following research projects:

1a. The membrane potential during the cell cycle

In synchronised cells the membrane potential is low during G_1 (-10 mV), rises during the S-phase, and reaches a maximum of ca. -50 mV in late G_2. Immediately after mitosis it returns to the low value. This suggests changes in the Na^+/K^+ permeability ratio during the cell cycle.

1b. The role of the membrane potential in the regulation of the cell cycle and the initiation of differentiation

The membrane potential can be specifically altered by changing the Na^+/K^+ ratio in the medium (the normal medium contains 116 mM Na^+ and 5.4 mM K^+). Using asynchronous, logarithmically growing cultures, changes in growth rate (^{3}H-thymidine incorporation, cell counting) and membrane potential as a function of external K^+ concentration were determined by replacing Na^+ in the medium by equimolar amounts of K^+. Growth is at a maximum at 30 mM K^+, declines at increasing K^+ concentrations, and stops at K^+ concentrations higher than 85 mM. The average membrane potential decreases with increasing external K^+ concentrations. When the membrane potential reaches values ≤ -10 mV (as in G_1 cells) growth stops: the cells are apparently unable to pass the transition from G_1 to S.

This effect is still completely and instantaneously reversible after 24 hrs. Preliminary results suggest that prolonged lowering of the membrane potential results in morphological differentiation (formation of neurites).

1c. The microviscosity of the plasma membrane during the cell cycle and in differentiating cells

This research was started in collaboration with Dr. M. Shinitzky at the Weizmann Institute of Science, Rehovot, Israel, where S. W. de L. stayed for some time twice during the year. The study is being continued at the Hubrecht Laboratory.

Microviscosity is a measure for the fluidity of the lipid bilayer of the plasma membrane and is determined quantitatively by introducing a carbohydrate probe molecule, 1,6 diphenyl-1,3,5 hexatriene (DPH) into the membrane and measuring its fluorescence polarisation. A method was developed for making these measurements on cells *in situ* in a monolayer. Morphological differentiation of neuroblastoma cells can be induced by exposing them to 1 mM dibutyryl cyclic AMP or to 10 μgr/ml prostaglandine E 1 + 200 μgr/ml Ro 20-1724 (a phosphodiesterase inhibitor). It was found that the membrane microviscosity decreases in differentiating cells.

The membrane microviscosity was also determined during the cell cycle in synchronised, growing cells: it is low during G_1, increases during the S-phase, and drops again during mitosis. A study was started of the effect of altering the membrane microviscosity on the cell cycle and differentiation. This is possible by bringing the plasma membrane into contact with artificial liposomes containing varying lecithine/cholesterol ratios.

Plasma membranes of logarithmically growing cells were isolated in different ways (see ii above) and their microviscosity was determined. Membranes isolated with the $ZnCl_2$ or latex method had the same microviscosity as that measured in intact cells. That of membranes of S.F. virus particles isolated from neuroblastoma cells was about twice as high.

Since these membranes have the same lipid composition as that of the host cell membrane, the results suggest that protein-lipid interactions within the membrane, or membrane geometry, or both, strongly affect the fluidity of the membrane.

Fluorescence polarisation can also be used to study the mobility of marker proteins specific for the plasma membrane of differentiating cells. It is known that the number of acetylcholine receptor sites in the membrane of neuroblastoma cells strongly increases during differentiation. In collaboration with Dr. M. Shinitzky (Rehovot) several fluorescent molecules were synthesised that bind specifically to acetylcholine receptors. They were tested for binding to both logarithmically growing and differentiating cells, and were shown to bind strongly to the latter. This method could be useful as a simple quantitative measure of the degree of molecular differentiation of the cells.

1d. Structural analysis of the plasma membrane

A beginning was made with a freeze-fracture EM study of the plasma membrane. Monolayers of cells are broken according to the method mentioned under iv above. The study is concerned with (1) changes in the structure of the membrane throughout the cell cycle, and (2) differences between logarithmically growing and differentiated cells.

1e. Cyclic nucleotide metabolism during the cell cycle and differentiation

A radio-immuno-assay for the determination of cAMP and cGMP has been elaborated. It was found that during differentiation of neuroblastoma cells (induced by PGL_1 + Ro 20-1724) their content of cAMP increases from ca. 10 to ca. 150 pM/mgr protein. Concentrations of cAMP and cGMP during the cell cycle are being studied, as is the influence of the membrane potential and membrane microviscosity on cyclic nucleotide metabolism.

2. *Freeze-fracture electron microscopy of the plasma membrane of the egg and early embryo (Xenopus laevis)* (J. G. Bluemink, L. G. J. Tertoolen, in cooperation with P. H. Ververgaert and A. J. Verkleij, Biological Ultrastructure Research Unit, University of Utrecht)

2a. The pre-existing and nascent membrane of the cleaving egg

During first cleavage in *Xenopus* new cell membrane is formed in the furrow region at a rate of ca. 4×10^4 μm^2/min. Freeze-fracture electron microscopy has produced the following data.

Pre-existing plasma membrane faces show a reversed polarity with respect to particle distribution, i.e., more particles are attached to the E-face (density 1600–2200 particles/μm^2) than to the P-face (300 particles/μm^2). A frequency histogram of 2331 measured intramembranous particles (E-face of pre-existing membrane) yields the following size distribution

(main classes only): 75 Å (2%), 95 Å (12%), 125 Å (30%), 180 Å (6%). At the tips of surface protrusions both the E- and the P-face are particle-free.

Intramembranous particles (∅ ca. 80 Å) found in other biological membranes are thought at least partly to represent proteins and/or glycoproteins complexed with lipids, which float in the lipid bilayer. The function of the 75–180 Å particles is unknown. They probably do not represent a class of proteins that facilitate the permeation of ions, because from previous studies at this Laboratory we know that the pre-existing membrane of the *Xenopus* egg is very impermeable (specific resistance 75 kOhm · cm²).

The observed reversed polarity of particle distribution has not been reported for cells of later amphibian embryos (Decker and Friend, 1974, *Rana pipiens* neurula). Further studies will be undertaken to elucidate if and when during development the polarity is reversed, and if so, whether this correlates with a change in membrane function, e.g. specific resistance.

Nascent cell membrane fracture faces are more difficult to obtain; they show a low particle density (300–500 particles/μm²). Lowering the ambient temperature to 5° C for 5 mins. does not alter the particle distribution but improves the output in nascent membrane fracture faces. From previous studies at this Laboratory it is known that the nascent membrane is highly permeable for ions (specific resistance 1.85 kOhm · cm²). The current view holds that intrinsic proteins traversing the thickness of the lipid bilayer may function as pathways for ions. The particles seen in the nascent membrane may represent such proteins, a class that would be lacking in the pre-existing membrane.

Alternatively, it is possible that nascent cell membrane still is a less perfect permeability barrier owing to a considerable degree of disorder of its lipids. Chilling would then reduce this degree of disorder, thereby improving the conditions for membrane splitting.

The fact that in the *Xenopus* egg pre-existing and nascent membrane regions are continuous and coexist while maintaining their highly different particle densities is noteworthy in itself. It provides evidence for a tangential structural stability which cannot easily be explained by the current dynamic fluid mosaic membrane model. This suggests the existence of a mechanism which can maintain a topographical heterogeneity in the cell surface; this might be the ultimate basis of the polarity of the fertilised egg.

2b. The pre-existing cell membrane in unfertilised and fertilised eggs

During the period preceding first cleavage the *Xenopus* egg undergoes alterations which may have a profound effect on the organisation of the cell membrane. In response to the event of fertilisation the cortical granules fuse with the cell membrane, their contents being extruded into the extracellular space. It is conceivable that the cortical granule membrane

is incorporated into the cell membrane. As a result, in this very early phase of development the particle pattern might undergo an alteration. To investigate this possibility, unfertilised eggs still having their full complement of cortical granules were freeze-fractured and the density of the particles associated with the E-face of the cell membrane was compared with that of the pre-existing membrane of cleaving eggs. No difference was found.

Normally 50–70 mins. elapse between the cortical reaction and the onset of cleavage. The possibility cannot be excluded that after the extrusion of the cortical granules the cell membrane would exhibit a transient change in particle pattern. This should be checked. The alternative possibility is that the membrane bounding the cortical granules does not become part of the cell membrane, the latter maintaining its original organisation. However, this would not be in accordance with the generally accepted view.

3. *The cell cycle of dissociated blastomeres (Xenopus laevis)*
(S. W. de Laat, P. T. van der Saag, C. M. A. W. Festen)

This work is the continuation of a project started last year (see previous report, sect. IV.5). Its aim was to analyse the possible relationships between membrane potential, cyclic nucleotide metabolism, and rate of DNA synthesis in blastomeres dissociated by incubation of the embryo in Sörensen's phosphate buffer.

Serious problems were encountered because the dissociation procedure drastically alters the intracellular ion concentrations. It is therefore impossible to distinguish between the effects of experimentally induced changes in the membrane potential (by varying the external Na^+/K^+ ratio) and those of the dissociation procedure *per se*.

VI. Other research projects

1. *Intracellular ion activities during salivary gland development (Chironomus thummi)* (H. H. Seydewitz, S. W. de Laat)

Puffing patterns in dipteran giant chromosomes respond in a specific manner to changes in nuclear electrolytes. It has been proposed (Kroeger, 1966) that controlled shifts in the activities of intracellular/intranuclear ions may play an important role in the activation and inactivation of genetic loci.

A culture of *Chironomus* was temporarily set up and a method was developed for measuring ion activities in isolated salivary glands by means of ion-selective micro-electrodes. The investigation is being continued in West Germany by H.H.S.

VII. Miscellaneous

1. Prof. R. Chandebois (Marseille) and Dr. T. Rogulska (Warsaw) stayed at the Laboratory for brief periods to use the library facilities.

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CENTRAAL BUREAU VOOR SCHIMMELCULTURES BAARN AND DELFT

Progress Report 1975

The Centraalbureau voor Schimmelcultures was founded in 1904 by the "Association Internationale des Botanistes". Dr. Johanna Westerdijk at Amsterdam was appointed as the first director in 1907. After the dissolution of the AIB, the Bureau was supported by various Netherlands scientific institutions and associations, esp. by the Royal Netherlands Academy of Arts and Sciences. In 1920 the institute moved to Baarn; the yeast collection has been kept since 1922 at the Laboratory of Microbiology, Technical University, Delft.

After Prof. Westerdijk retired in 1959, she was succeeded as director by Miss A. L. van Beverwijk (1959–1963). In 1964 the CBS moved into a new building in Baarn (Oosterstraat 1). Since 1968, the CBS has been an institute of the Royal Netherlands Academy of Arts and Sciences.

The Centraalbureau voor Schimmelcultures maintains a collection of living fungi, yeasts and actinomycetes. In 1975 the total number of strains maintained was 22.000, including 3.900 yeasts. By supplying cultures, identifications and advice to workers in diverse fields of scientific and applied mycology, a service is rendered to all those interested in these micro-organisms. Scientific research is carried out mainly in taxonomy of fungi. In the division of human and animal mycology, problems pertaining to this field are studied. Investigations on the chemistry of fungal metabolites are carried out in the biochemical department.

Facilities are available to students and guest workers who want to study a mycological subject. Each year courses are given on general and on human and animal mycology.

Scientific Staff (as from December 1st 1975).

Baarn, Oosterstraat 1.

Dr. J. A. von Arx, director (general mycology, Ascomycetes, Melanconiales)
Dr. G. A. de Vries (human and animal mycology, Actinomycetes)
Miss dr. M. A. A. Schipper (Mucorales)
Mrs. drs. E. J. Hermanides-Nijhof (Fusarium, Aureobasidium)
Mrs. drs. A. J. van der Plaats-Niterink (Oomycetes)
Dr. H. A. van der Aa (Sphaeropsidales)
Dr. G. W. van Eijk (biochemistry)
Dr. W. Gams (Verticillium, Acremonium and related genera, Mortierella)
Dr. R. A. Samson (Paecilomyces, Penicillium and related genera)
Dr. G. S. de Hoog (Dematiaceae)
Drs. J. A. J. M. Stalpers (Basidiomycetes)
Drs. A. C. M. Weijman (biochemistry, physiology)
Miss C. A. N. van Oorschot, M. Phil. (Chrysosporium and allied genera)
Mrs. drs. G. de Bruin-Brink (documentation)

Yeast Division, Delft, Julianalaan 67A.

Prof. Dr. T. O. Wikén, Director Laboratory of Microbiology, Technical University
Drs. L. Rodrigues de Miranda (Basidiomycetous yeasts)
Miss drs. M. Th. Smith
D. Yarrow (Saccharomyces and related genera)

Introduction

In recent years several techniques have become available which complement light-microscopic morphological studies on fungal taxonomy. These developments must no longer be neglected. Morphological details can be greatly elucidated by means of electron microscopy; the scanning electron microscope provides a particularly rapid means of obtaining a detailed, three-dimensional impression of almost undisturbed fungal cells. The analysis of structures by electron microscopy leads to a better understanding of light-microscopic observations and enhances ontogenic interpretation.

Genetics contributes the soundest basis for species delimitation. The simplest phenomenon is interfertility between strains and it is used as a criterion of conspecificity. This information is still of undiminished importance when judging morphological variability within species.

Groups with little morphological differentiation may sometimes be elucidated by chemical investigations. Important information regarding a large-scale arrangement of different fungi can be obtained from the analysis of cell walls. The GC content of DNA is most commonly determined in the chemical analysis of genetic material. This technique has shown the heterogeneity of some genera of yeasts and oomycetes and the GC content can now generally be used in characterizing newly described organisms. Chemical "finger-printing" of whole organisms has been attempted by various means. Gas chromatography of pyrolysis products provides a pattern of peaks which are difficult to interpret and to reproduce. The combination of pyrolysis with mass spectrometry, however, opens some important perspectives. The combination of morphologic, genetic and chemical data, leads to the establishment of a potentially stable classification. As the data become increasingly complex and difficult to assess, a computer may be used to assist the sorting of taxa and the selection of crucial features.

The results obtained in 1975 are given below under the names of the coworkers studying mainly the respective group of fungi within each division.

1. Division of Fungus Taxonomy

Dr. H. A. van der Aa

The study of the type specimens of *Phyllosticta* species was continued. Large series were obtained from the herbarium of the State University of Utrecht and the herbarium of P. A. Saccardo in Padua. From the Botanische Staatssammlung in München the whole collection of species described by A. Allescher was received for study. With regard to species of *Phyllosticta* sensu stricto, the following additions to the first paper may be given (Van der Aa, 1973: Stud. Mycol. 5).

Phyllosticta codiaei Sacc. was already regarded as a synonym of *Phyllosticta ghaesembillae* Koorders on the basis of the description. This could now be confirmed by a study of the type specimen which also contained the as yet unknown *Leptodothiorella* spermatial state. *Phyllosticta circumsepta* Sacc. was found to be a further synonym of *Phyllosticta citricarpa* (McAlp.) van der Aa. *Phyllosticta cocoina* Sacc. was found to be the oldest name for the conidial state of *Guignardia calami* (Syd.) v. Arx & Müller. In the type specimen the as yet unknown *Leptodothiorella* state was also found. *Phyllosticta ericae* Allescher and *Phyllosticta ericicola* Died., both based on the same collection, proved to be synonyms of *Phyllosticta pyrolae* Ellis & Everh. and the *Leptodothiorella* state from the same life cycle. The ascigerous state, probably a *Guignardia* species, was observed in an immature state.

Numerous other *Phyllosticta* species turned out to be synonyms of well known *Phoma* or *Coniothyrium* species or the *Asteromella* state of *Mycosphaerella* species.

In co-operation with Drs. G. H. Boerema (Plantenziektenkundige Dienst, Wageningen) a new *Didymella* species and its *Phoma* conidial state were studied. This species was repeatedly collected in the Netherlands on leaf spots and on living and dead stems of *Urtica dioica*. Also in co-operation with Drs. Boerema a comparative study was started of the *Phoma* and *Ascochyta* species which occur on *Chenopodium* and *Atriplex* species. For these fungi numerous names in *Phoma*, *Phyllosticta*, *Ascochyta* and some more genera are available. From cultural studies and the study of type specimens it is clear that the number of names can be greatly reduced.

On twigs of *Buxus sempervirens*, collected on Mont Ventoux in South France, two undescribed ascomycetes were discovered and isolated. The first one was a *Guignardia* species with 16- or 32-spored asci, isolated from three different ascomata. These all give well sporulating cultures of *Sarcophoma miribelii* (Fr.) Höhnel. It was concluded that this state belongs to the same life cycle, and the ascigerous state was described as *Guignardia miribelii* spec. nov. Isolation of *Hyponectria buxi* (Desm.) Sacc. was attempted from freshly collected material. Several previous authors had suggested that this might be the ascigerous state of *Sarcophoma miribelii*. However, several attempts to germinate the ascospores were

unsuccessful and hence a relation to any conidial fungus could not be proved. The results of these studies have been published in Persoonia.

On the same collection of twigs of *Buxus sempervirens* an undescribed species of *Paraphaeosphaeria* was found, producing a *Coniothyrium* conidial state in culture. Both the ascomata and the pycnidia have well developed papillae covered by characteristic orange crystals. The fungus will be described at a later date.

The type culture of *Leptosphaeria elaeidis* Booth & Robertson (Trans. Br. mycol. Soc. *44*: 24, 1961), (CBS 413.62 = IMI 61175) proved to represent a further species of the genus *Pestalosphaeria* Barr (Mycologia *67*: 187, 1975), and has to be classified as *Pestalosphaeria elaeidis* (Booth & Robertson) van der Aa, comb. nov. The asci are unitunicate and have an amyloid apical ring. *Pestalosphaeria concentrica* Barr is morphologically similar but can be distinguished by a deviating *Pestalotia* conidial state.

The new genus *Echinochondrium* was described in collaboration with Dr. R. A. Samson. The only species, *E. pulchrum*, was isolated from a dead leaf of *Cocos nucifera* collected in Ceylon by Dr. W. Gams. The fungus was compared with several similar genera, especially with *Dactuliophora* Leakey, but it differs by the absence of sclerotiophores. The sclerotial bodies of *Echinochondrium* originate from initials which enlarge without the differentiation of supporting structures.

Dr. J. A. von Arx

The revision of the ascomycetes sporulating in pure culture was continued. The CBS strains maintained under the names *Lophotrichus* and *Kernia* were examined. In all typical species of the latter genus the ascospores have a single germ pore. The genus is close to *Microascus*, as re-defined by von Arx (Persoonia *8*: 191–197, 1975), and intermediate forms exist. The genus *Lophotrichus* can be distinguished by the somewhat larger ascospores with two distinct germ pores, one at each end; by ostiolate, rarely non-ostiolate, thin-walled, hairy ascomata and by the absence of a conidial state. The "species" described in *Lophotrichus* are close to each other and will probably have to be classified as a single species, *L. bartletii* (Massee & Salmon) Malloch & Cain. *L. incarnatus* Seth has to be excluded, as it is identical with *Chaetomium cruentum* Ames, a rather peculiar ascomycete with fleshy, pale ascomata and large, pale ascospores.

Cultures of some recently described *Thielavia* species became available. *Thielavia angulata* Das (Trans. Br. mycol. Soc. *45*: 545, 1962) proved to represent a new ascomycete genus of uncertain relationship, for which the name *Eremodothis* was proposed. In the course of this study some more ascomycete genera had to be compared. The fungus described as *Bitrimonospora indica* Sivanesan & al. (Trans. Br. mycol. Soc. *63*: 595, 1975) proved to be identical to *Rechingeriella eutypoides* Petrak (Sydowia *8*:

170, 1954) and was transferred to the genus *Monosporascus*. *Thielavia inaequalis* Pidoplichko & al. (Mikrobiol. Zh., Kiev *35*: 723, 1973) was found to represent a further species of the genus *Corynascella* v. Arx (Stud. Mycol. *8*, 1975). The same fungus was again described as *Thielavia wareingii* Seth (Nova Hedwigia *25*: 468, 1974). *T. pallidospora* Pidoplichko & al. proved to belong to *Petriellidium ellipsoideum* v. Arx & Fassatiová (Persoonia *7*: 370, 1973). A few more strains of this species became available, some having been isolated from marine substrates.

The study of the mycelial yeasts was continued, partly in co-operation with Miss Drs. M. Th. Smith (Delft) and Dr. J. P. van der Walt (Pretoria, South Africa). All species classified in the genus *Saccharomycopsis* were compared. The heterothallic mycelial yeast *Pichia burtonii* Boidin & al. (=*Endomycopsis burtonii* (Boidin & al.) Kreger-van Rij) can be classified neither in *Pichia* nor in *Endomycopsis (Saccharomycopsis)*, but has to be transferred to an as yet undescribed genus. Its conidial state is *Sporothrix*-like (De Hoog, Stud. Mycol. 7, 1974) with denticulate conidiogenous cells and small apiculate, often catenulate conidia. The asci are formed exclusively by conjugating yeast cells and usually contain one or two pairs of small, hat-shaped ascospores, 2–3.5 μm in diameter. In typical species of the genus *Saccharomycopsis* the asci are formed in lateral, terminal or intercalary position on diploid hyphae, the ascospores being larger, usually 5–8 μm in diameter and the conidia usually elongated, with a truncate base and leaving scars.

The re-evaluation of the bitunicate ascomycetes, written in co-operation with Prof. E. Müller (Zürich) was published in Studies in Mycology No. 9. A single order, Dothideales, is accepted and contains 34 families (with 26 synonyms) and approximately 425 genera. More than 600 superfluous generic names are enumerated in the synonymy, about 145 of them for the first time.

A short manuscript was prepared concerning taxonomic work in a cul ure collection of fungi.

Dr. W. Gams

The work on an expanded version of the book on soil fungi was continued together with Prof. K. H. Domsch and Mrs. H. Anderson (Braunschweig, F.R.G.). The taxonomic sections, including keys to the selected species, were written for the larger genera *Aspergillus* and *Penicillium* (including perfect states), *Acremonium*, *Trichoderma* and *Mortierella*. All recognizable species of the latter genus were keyed out in an arrangement similar to that published by Gams in 1970 (Nova Hedwigia *18*: 30–43). In order to confirm some suspected synonymies, mating experiments were carried out which yielded zygospores between *M. spinosa* Linnem. and *M. mutabilis* Linnem. and between *M. elongata* Linnem. and *M. rishikesha* Mehrotra & Mehrotra. The zygosporic states recently described by Kuhlman (Mycologia

67: 678–681, 1975) were reproduced and confirmed. The agar made from "Pablum" cereals used by that author was successfully replaced by a similar product from "Bambix".

The work on phialidic dematiaceous Hyphomycetes which grow on decaying wood is nearing completion. It is centred around the genus *Chloridium* (integrated conidiogenous cells, one-celled conidia), and the similar genus *Cylindrotrichum* (2-celled conidia), but will also comprise several *Phialophora* species (generally discrete conidiogenous cells) with catenulate conidia, and two dark species, formerly placed in *Verticillium*, for which a new genus will be proposed. A large collection of well preserved herbarium specimens from Dr. V. Holubová-Jechová (Pruhonice, Czechoslovakia), contained several new species and also often the appropriate perfect states of *Chaetosphaeria*. A further important collection of specimens was obtained from Prof. G. L. Hennebert, Louvain-La Neuve. The generic concepts provide some problems: The genus *Chaetosphaeria* will have to be expanded so as to include species with fragmenting ascospores similar to *Trichosphaerella*, but with non-setose ascomata (perfect state of *Chloridium chlamydosporum*). In *Chloridium* at least four distinct sections can be recognized: species with a very short collarette at the phialide tip can be subdivided into a group showing a regular occurrence of sympodial elongation (polyphialides, e.g. *C. minutum*) and one showing only percurrent elongation. In the latter group species with a single conidiogenous locus and species with multiple, adjacent conidiogenous loci can be distinguished. Preuss's genus *Gongromeriza*, the conidial state of *Chaetosphaeria myriocarpa* (Fr.) Booth, is characterized by a long, distinctly pigmented collarette. As species of all groups were found to have perfect states in *Chaetosphaeria*, it is preferable to classify them in a single genus *Chloridium*.

The work on *Niesslia* ascigerous states and *Monocillium* conidial states progressed with the revision of some of Spegazzini's *Venturia* species which provided valid epithets for some European species. Keys to 22 perfect (6 new) and 23 conidial states (including some aggregates, 6 new) are in preparation.

Phialophora parasitica Ajello & al., recently described from a subcutaneous infection in man, was recognized as a causal agent of wilt in *Nectandra* sp. in Costa Rica and in *Phoenix dactylifera* in Iraq. A comparative study of strains of different origin was prepared for publication in the Transactions of the British Mycological Society jointly with Drs. D. L. Hawksworth and I. A. S. Gibson (C.M.I., Kew). In collaboration with Dr. Hawksworth a new genus *Sarocladium* was described to accommodate the rice pathogen *Acrocylindrium oryzae* Sawada and a related species which both have dense, broom-like clusters of phialides on irregularly branched conidiophores. The paper will be published in Kavaka (Madras, India).

Mrs Drs. E. J. Hermanides-Nijhof

The revision of the fungi classified in *Aureobasidium* and in some other genera belonging to the so-called "black-yeasts" was continued.

The genus *Aureobasidium* has to be restricted to species with conidia which are formed simultaneously; so far only one species, *A. pullulans* (de Bary) Arnaud, can be maintained.

All other species described in this genus have to be excluded since the conidia are produced in basipetal succession or in sympodulae. For example, *Aureobasidium bolleyi* (Sprague) v. Arx is characterised by falcate conidia formed singly or sympodially directly on the hyphae or on ampulliform conidiogenous cells. This species will be classified in a new genus. *Aureobasidium foliicola* (Oudem. ex Delitsch) G. Müller produces conidia in basipetal succession on intercalary or lateral conidiogenous cells. Although the conidium production is similar to that of *Hormonema* species, other characters are very different. *Aureobasidium foliicola* never blackens, but remains orange in colour. In *Aureobasidium prunorum* Dennis & Buhagiar the conidia are also formed in basipetal succession; this species will thus be placed in the genus *Hormonema*.

A morphological study of *Pullularia werneckii* (Horta) de Vries (= *Cladosporium werneckii* Horta) was begun. This species does not belong to either the genus *Aureobasidium* (= *Pullularia*) or to *Cladosporium*. The often two-celled conidia seem to be formed on indistinct sympodulae. Strains which sporulate abundantly are yeast-like, whilst others form a greyish-green mycelium. This fungus has to be compared with *Exophiala salmonis* Carmichael, the type-species of the genus *Exophiala*, in which it was classified by von Arx (The Genera of Fungi Sporulating in Pure Culture, p. 180, 1970).

The study of available herbarium specimens or of cultures of other species described as *Aureobasidium* or *Pullularia* is in progress.

The genus *Hormonema*, characterized by the production of conidia in basipetal succession on often intercalary, phialide-like cells, includes mainly the "black-yeast"-like cultural states of ascomycete genera such as *Dothiora*, *Pringsheimia* or *Sydowia*. The species delimitation in culture is difficult, differences being found in growth rate of the colonies and in temperature requirements.

A study of other species mentioned as synonyms of *Aureobasidium pullulans* by Ciferri & al. (Atti Ist. Bot. Lab. Crittog. Pavia *14:* 78–90, 1956) and Cooke (Mycopath. Mycol. appl. *17:* 1–43, 1962), for example *Cryptococcus nigricans* Rich. & Stern, is nearly completed.

Dr. G. S. de Hoog

The morphology of over 120 strains of black yeasts was studied in detail. For each strain a list of about 150 character notations was prepared in order to process the data by means of a computer. Special attention

was paid to the conidium ontogeny. In addition to budding cells either phialidic, annellidic or sympodial conidiogenesis occurs, or sympodial cells become prevalent in older annellidic strains. In a few cases the fertile cell starts forming conidia percurrently, at a later stage becoming sympodial. These strains are reminiscent of phialidic *Chloridium* species. In many *Rhinocladiella*-like strains budding cells are absent or very rare. The infrequent occurrence of phialides which liberate conidia by deliquescence of the apical part of the cell, in strains with annellidic and/or sympodial conidiogenesis, as well as in *Rhinocladiella compacta*, indicate that the species under consideration may be more closely related than can be concluded from superficial morphological study. Hence "finger-prints" of the chemical composition of each strain are now being analysed by means of pyrolysis mass spectrometry, in co-operation with Dr. H. L. C. Meuzelaar (F.O.M.-Institute, Amsterdam).

Where available, original material has been studied of almost all species assigned to the group. A list of 15 doubtful, invalid or impracticable genera, containing over 60 species, has been compiled. Some imperfectly known species have been redescribed.

A few miscellaneous Hyphomycetes have been described in a separate paper: a new species of *Sympodiophora*, and a new genus, which is close to *Sporothrix* but forms conidia more or less retrogressively on small pegs in the apical portion of the conidiogenous cell.

Miss C. A. N. van Oorschot

A survey was made of the available literature on *Chrysosporium* Corda, the related genera *Emmonsia* Cif. & Montem., *Malbranchea* Sacc. and *Myceliophthora* Cost., and also of the ascomycetous states of *Chrysosporium* species. References and/or descriptions of a total of 35 different *Chrysosporium* species were found. Carmichael (Can. J. Bot. *40*: 1137–1173, 1962) in a preliminary monographic treatment recognized 9 species. In 1967 Dominik (Zeszyty naukowe wys. Szk. Roln. Szczecin. Ser. 3, *24*: 37–66) constructed a key to 20 species of *Chrysosporium* and a number of new species have subsequently been erected by different authors. A detailed study of all known species of *Chrysosporium* and of related genera is overdue and a new basis for genus and species delimitation may have to be established.

Preliminary observations have been made of representative strains of the *Chrysosporium* species in the CBS collection on malt and oatmeal agars at 21° C to allow comparisons to be made under defined conditions. A few undetermined *Chrysosporium* species are also being investigated. Conidial size and ontogeny appear to be the most distinguishing features thus far.

Drs. A. J. van der Plaats-Niterink

In the course of the year a monograph of the genus *Pythium* was initiated and this will be the most important task for the coming years. One of the subjects for this study was a group of closely related species which produce swollen toruloid sporangia: *P. torulosum*, *P. vanterpoolii* and *P. dissimile*. These species differ mainly in the origin of the antheridia; 3–10 μm below the oogonium in *P. torulosum* and about 25 μm below the oogonium in *P. vanterpoolii*. In *P. dissimile* the antheridia are mostly lacking. There are also differences in daily growth rate, maximum temperature resistance and diameter of oogonia. In young cultures the sporangia seem to be of a non-swollen type. Later the typical swollen toruloid sporangia appear, while the production of zoospores continues or even increases. In the Netherlands *P. vanterpoolii* has not been isolated and *P. dissimile* only from one site, but *P. torulosum* occurs rather frequently, preferring soils from gardens, pastures and golf-links.

Within the group with non-swollen filamentous sporangia 12 strains of *P. coloratum* sent by Dr. O. Vaartaja were studied and compared with related species, e.g. *P. dissotocum*. These two species show a great resemblance in morphology: thick-walled oospores, smooth oogonia, diclinous and monoclinous antheridia. The sporangia in young cultures have the same appearance as the vegetative hyphae and in older cultures they consist of complexes of slightly swollen, rectangularly branched, but never toruloid filaments. *P. coloratum* differs from *P. dissotocum* by smaller, mostly terminal oogonia, and by usually diclinous antheridia with rather long, sometimes branched stalks. In *P. dissotocum* most oogonia are intercalary and diclinous as well as monoclinous, sac-like antheridia are present. Other differences can be found in the cultural characteristics, the daily growth rate and the temperature requirements.

In the group of species with ornamented sporangia, *P. megalacanthum* sensu Buisman was studied. Many attempts to force this strain to produce sporangia failed. The species, only isolated from flax or from soil of flax fields, is certainly different from *P. megalacanthum* de Bary and needs renaming.

In co-operation with Dr. R. A. Samson, Drs. J. A. Stalpers and Drs. A. C. M. Weijman a number of species producing ornamented, spherical, asexual reproductive organs were studied. After a morphological study, several crossings and many experiments with different media, most isolates could be placed in the genus *Mortierella*. The genus *Azygozygum* is considered to be a chlamydosporic state of *Mortierella*.

Dr. R. A. Samson

A manuscript entitled "A revision of the subsection *Fasciculata* of *Penicillium* and some allied species" was prepared in collaboration with Drs. A. C. Stolk and Prof. R. Hadlok (Giessen) and will be published

as Studies in Mycology no. 11. In this paper the species classified by Raper and Thom (1949) in the subsections *Fasciculata*, *Lanata* and *Funiculosa* are treated. The study is based on type cultures and on hundreds of strains mostly isolated from food products. A new species delimitation is proposed based mainly on morphological characters of the conidiogenous structures. Nineteen taxa, including three new ones are proposed. The name *Penicillium verrucosum* Dierckx (1901) is reintroduced to include the morphologically identical species of the *P. ochraceum*, *P. viridicatum* and *P. cyclopium* series and partly those of the *P. expansum*, *P. granulatum*, *P. commune* and *P. terrestre* series. To distinguish macroscopically recognizable groups within *P. verrucosum*, the varieties *album*, *corymbiferum*, *cyclopium*, *melanochlorum* var. nov. and *ochraceum* are proposed. The accepted species and varieties are illustrated and described, synoptic and dichotomous keys being given.

The re-examination of the taxonomy of the *Penicillium chrysogenum*-series was continued. On account of the observations of the available type strains and numerous isolates mainly obtained from food products, *P. notatum*, *P. meleagrinum* and *P. cyaneofulvum* are placed in synonymy with *P. chrysogenum*. A statistical analysis of the conidium size and shape in about 90 strains was carried out. A manuscript in collaboration with Prof. Dr. R. Hadlok and Drs. A. C. Stolk is in preparation.

A compilation of the *Aspergillus* species described since 1965 was started. About 60 new taxa have been described, after the monograph of the genus *Aspergillus* was published by Raper and Fennell (1965). The available type strains were studied and compared with those of the related species to clarify their identity.

Together with Drs. J. A. Stalpers the ornamentation of the zygospores of the genera *Mucor* and *Zygorhynchus*, provided by Dr. M. A. A. Schipper, were examined by means of scanning electron microscopy. From these observations it was shown that three groups of ornamentation type could be distinguished. This grouping correlates well with the species classification based on the morphology of the asexual state.

With support of a Nato Science Fellowship awarded for the period November 1975 to September 1976 by the Dutch Organization of pure scientific research, the ultrastructural studies on conidium ontogeny in the Hyphomycetes are continued at the Department of Botany of the University of Texas at Austin (USA). This project, in collaboration with Dr. G. T. Cole (Austin, USA), Dr. W. Gams and Drs. J. A. Stalpers aims at an illustration of the various types of conidium ontogeny in the Fungi Imperfecti by means of various available techniques. The results will be published in an atlas illustrated with SEM and TEM micrographs including a compilation of the literature on this subject.

Dr. M. A. A. Schipper

A manuscript was prepared on *Mucor circinelloides*, *M. racemosus* and related species, which will be published as Studies in Mycology, No. 12, 1976. The methods described were those used in former studies; similarity and interfertility again being used as the main criteria indicating relationship.

Mucor circinelloides (and its formae *lusitanicus*, *griseo-cyanus* and *janssenii*), *M. ramosissimus*, *M. bainieri* and *M. zonatus* show a general morphological resemblance, whereas *M. racemosus* (and its formae *sphaerosporus* and *chibinensis*), *M. plumbeus* and *M. fuscus* form another natural group. Interfertility tests confirmed that *M. circinelloides*, *M. lusitanicus*, *M. griseocyanus* and *M. janssenii* can be classified in a single variable species, as mature zygospores were obtained from matings with each other. Though *M. racemosus* and *M. plumbeus* were also able to produce zygospores on matings, these zygospores seemed to remain immature. In matings of *M. circinelloides* with *M. racemosus* and with *M. plumbeus*, similar types of probably immature zygospores were obtained.

The zygospores of *M. plumbeus*, first observed by Bainier (1884) but never since, were obtained in matings of typical *M. plumbeus* strains, each of the (−) mating reaction type, with some recent isolates with smooth columellae, of the (+) reaction type.

In cooperation with Dr. W. A. Shipton (Townsville, Australia) a paper was published on "Halteromyces, a new genus in the Mucorales"; with Prof. R. Hadlok (Giessen, F.R.G.) entitled "Schimmelpilze und Fleisch: Reihe Mucorales"; with Dr. R. A. Samson & Drs. J. A. Stalpers on "Zygospore ornamentation in the genera *Mucor* and *Zygorhynchus*".

A short communication was prepared on the production of azygospores in *Mucor (Rhizomucor) pusillus* strains contrasted with *Absidia corymbifera* (=*A. ramosa*) strains. The azygospores were of the *M. pusillus*-type. No visible reaction was found in *Absidia corymbifera*.

Drs. J. A. Stalpers

Basidiomycetes with aleurioconidia borne singly or in chains are classified in *Sporotrichum* and *Ptychogaster* respectively. Perfect states of these genera belong to the Corticiaceae and the monomitic Polyporaceae (*Phanerochaete*, *Abortiporus*, *Oligoporus*). The genus *Sporotrichum* may be subdivided into 2 groups: the first with relatively wide hyphae which may be thick-walled, with rare or absent clamp-connections and with large chlamydospores (e.g. *S. aureum* Link ex S. F. Gray, *S. pulverulentum* Novobranova), the second with relatively narrow hyphae which are thin-walled, with clamp-connections at all primary septa and without chlamydospores (e.g. *S. azureum* Wright & v. Arx, *Abortiporus biennis* (Bull. ex Fr.) Singer).

Up to now, the only chemical tests normally used for descriptions of

cultures of Basidiomycetes, are those on laccase and tyrosinase. Recently, J. B. Taylor (Ann. appl. Biol. *78*: 113–123, 1974) described a number of instant tests and incubatory tests on several enzymes produced by basidiomycetes, e.g. cytochrome oxidase, lecithinase, lipase, fucosidase. Some experiments have been started to find out if the presence of these enzymes is a reliable taxonomic character, i.e. if it is specific for species or larger taxonomic entities. It is being attempted to try to modify some of the incubatory tests so that they can be used as instant tests.

A manuscript comprising descriptive keys to the resupinate non-poroid Aphyllophorales in Europe, USSR and North America in collaboration with Dr. W. Jülich is nearly completed. For this purpose some genera have been revised, among which *Mycoacia* Donk, a genus with hydnoid ceraceous basidiocarps. It is concluded, that differences in a mere colour reaction with KOH only are not sufficient for species delimitation. Therefore, *M. uda* (Fr.) Donk, *M. fuscoatra* (Fr. ex Fr.) Donk and *M. kurilensis* Parmasto can only be separated at the form level. Species without clamp-connections (*M. squalina* (Fr.) M. P. Christ.) and species with skeletal hyphae (*M. denticulata* sensu Bourdot & Galzin) are not congeneric.

The genus *Coniophora* is redefined and comprises only species with multiple clamp-connections and multi-nucleate cells. Several species with single clamp-connections at nearly all septa and with dikaryotic cells, formerly placed in *Coniophora* because of their yellowish, thick-walled spores and their large basidia, are now referred to the genus *Leucogyrophana* Pouzar.

The genera *Thanatephorus* Donk, *Uthatobasidium* Donk and *Ypsilonidium* Donk are combined under the first name because of the lack of stable differentiating characters; e.g. *Ypsilonidium* is erected for species with two-spored basidia. However, a specimen of *Uthatobasidium fusisporum* (Schroet.) Donk contained areas where the basidia bore one, two or three to four sterigmata, respectively. *Thanatephorus* contained originally parasitic species with *Rhizoctonia*-type hyphae and with or without sclerotia, but some modern authors also add *Ceratobasidium anceps* (Bres. & Sydow) Jackson, which is not *Rhizoctonia*-like, whilst some authors also consider the species of *Ypsilonidium* (saprophytic, without sclerotia, not *Rhizoctonia*-like) to belong here.

2. Division of Biochemistry and Physiology

Dr. G. W. van Eijk

The presence of long white crystalline needles was observed in 6–10 month old cultures of *Mycena megaspora* CBS 363.50. About 1 mg crystals was collected and purified by recrystallisation from acetone. The compound

dissolved in ethanol showed aromatic absorptions in ultra-violet light. The mass spectrum revealed molecular ion peaks characteristic of a substance with 4 chlorine atoms in its molecule. High resolution mass spectrometry gave a molecular formula $C_8H_6Cl_4O_2$. The fragmentation pattern was identical with that described for drosophilin A *O*-methylether (1,4 dimethoxy-2,3,5,6 tetrachlorobenzene), a compound isolated from 2 *Fomes* species. The identity of the *Mycena* metabolite as drosophilin A *O*-methylether was proved by comparing it with a synthetic sample. There was complete agreement in all respects (m.m.p., IR and UV).

The same type of crystals were observed in cultures of a *Stereum* species. At first the presence of the same metabolite mentioned above was suspected. However, remarkable differences were detected in the infra-red and ultraviolet spectra of both compounds. The results obtained so far indicate that the *Stereum* metabolite may be a long-chained unsaturated alcohol or a sesquiterpene alcohol.

The pigments from *Pseudospiropes simplex* CBS 675.74, a dematiaceous hyphomycete, were studied. The major product was obtained as orange-red crystals. Its infra-red spectrum showed bands at 1742 cm^{-1} characteristic for CO, arising from a normal ester or an α, β-unsaturated γ-lactone, at 1660 and 1620 cm^{-1} due to the presence of a free quinone group and a hydrogen-bonded quinone group, respectively. The mass spectrum showed a molecular ion at 256.0365 in agreement with $C_{14}H_8O_5$. This formula ruled out a normal anthraquinone derivative. Therefore a naphthoquinone structure was presumed and a search of the literature revealed such a compound with similar physico-chemical properties to those of the metabolite. This compound is the pigment lambertellin, only previously isolated from several species of the inoperculate discomycete *Lambertella*. A generous gift of synthetic lambertellin from Prof. R. H. Thomson enabled us to compare both compounds with each other. It could be unambiguously ascertained that the isolated compound was indeed lambertellin. A minor metabolite seemed to be chrysophanol, a well-known anthraquinone pigment. A minute amount of a third substance formed, was probably also an anthraquinone derivative. Its structure is still under investigation. The production of lambertellin by *P. simplex* is surprising as there does not exist any taxonomic relationship between this fungus and *Lambertella*. Furthermore the co-occurrence of lambertellin together with chrysophanol is also most interesting in view of the biogenetic origin of the former compound. It supports Turner's hypothesis (W. B. Turner, Fungal Metabolites p. 196, Academic Press, London, 1971) that lambertellin might arise by degradation of chrysophanol.

A yellow crystalline metabolite was obtained from 2% malt agar cultures of *Monodictys castaneae* CBS 666.74. The ultra-violet spectrum of the compound showed the absorption peaks (329 and 400 nm) typical of an α-hydroxy-anthraquinone. The infra-red spectrum also indicated this structure, peaks being observed at 1672 cm^{-1} (free CO) and 1640 cm^{-1}

(chelated CO). These results combined with mass spectroscopic data suggested that the isolated substance was pachybasin. This was proved by comparing the metabolite with synthetic pachybasin obtained by condensation of phthalic acid anhydride with *m*-cresol. Another metabolite of *M. castaneae* could be identified as chrysophanol. The quantities of some other pigments isolated were too small to be completely analysed.

Drs. A. C. M. Weijman

The study of the taxonomic application of fungal carbohydrate constitution, especially of yeast-like fungi, was continued and extended to 168 strains. The "intact cell" approach greatly enhanced the chemotaxonomical applications. As a result a study could be published in cooperation with Dr. G. S. de Hoog, titled "On the subdivision of the genus *Ceratocystis*". This subdivision in *Ceratocystis* sensu stricto and *Ophiostoma* was based on conidium ontogeny and rhamnose distribution. The genus *Ceratocystis* was restricted to species with phialoconidia and cell walls without rhamnose, whereas the genus *Ophiostoma* comprises species with exoconidia and rhamnose in the cell wall.

The hypothesis concerning a relationship between *Cephaloascus fragrans* and members of the Ophiostomataceae, based on similarities between the conidiophore of the *Verticicladiella* state of *Europhium aureum* and the ascophore of *Cephaloascus fragrans* (Cain, Mycologia *64*: 1–14, 1972), is not confirmed by examination of rhamnose distribution. All strains of *Cephaloascus fragrans* maintained in the CBS collection lack rhamnose. From literature it is known that cellulose is a component of the cell wall, of *Ophiostoma* (including *Europhium*) (Rosinski and Campana, Mycologia *56*: 738–744, 1964; Jewell, Mycologia *66*: 139–146, 1974). Therefore it seemed worthwhile to check these results and to study the possible occurrence of cellulose in *Cephaloascus* in order to reject or confirm the hypothesis mentioned above. Cellulose could not be detected in *Cephaloascus fragrans*, whereas in *Europhium aureum* only a trace could be demonstrated. On the basis of cellulose and rhamnose distribution there is no reason to support Cain's hypothesis.

The intact cell technique was also applied in a study of Oomycetes and Zygomycetes which produce only ornamented reproductive structures (conidia, chlamydospores). This study was carried out in cooperation with Mrs Drs. A. J. van der Plaats-Niterink (taxonomy), Dr. R. A. Samson and Drs. J. A. Stalpers (scanning electron microscopy). Fungi belonging to the Oomycetes (*Pythium*, *Trachysphaera*) have a high glucan and a low glucosamine content, whereas members of the Zygomycetes (*Mortierella*, *Azygozygum*) show a high glucosamine content and possess fucose and glucuronic acid.

Cooperation was started with Dr. H. L. C. Meuzelaar (F.O.M. Institute, Flash Pyrolysis Group, Amsterdam), in order to study the possibilities

of the new technique of Pyrolysis Mass Spectrometry (Py-MS) in fungal taxonomy. Py-MS can be applied to the characterization of complex biological materials as, for example, fractions obtained during the extraction procedure of cellulose and other glucans (Meuzelaar et al., Biomed. Mass Spectrom. *1*: 312–319, 1974). However the most important application of Py-MS is the fingerprint analysis of strains in combination with a multivariant analysis carried out with the aid of a computer. Results are plotted on a non-linear map showing relationships between the strains based on significant peaks in the spectra. The Py-MS analysis of yeast-like Ascomycetes, including members of the genera *Ascoidea*, *Endomyces*, *Saccharomycopsis*, *Ambrosiozyma* for example is in progress.

3. Division of Medical Mycology

Dr. G. A. de Vries

Since the discovery of Lobomycosis in 2 Bottle-nosed dolphins in the Dolphinarium at Harderwijk in August 1974 no other cases have been reported. A suspected case in a specimen of the fresh water dolphin (*Inia geoffroyi*) was etiologically different. The skin material of this animal was kindly put at our disposal by Prof. Dr. J. J. Laarman. The ascomycete *Petriella setifera* which had been isolated from the infected skin of *Sotalia guianensis* and the Harderwijk *Tursiops truncatus* specimens could also be grown from the pool water samples in which the diseased Bottle-nosed dolphins were kept. The approximate concentration of the fungus in the water calculated from the results obtained with the ultracentrifuge and filter technique was found to be 11 spores (or other viable units) per liter. Whether the fungus originated from the skin of the dolphins or from some other source was not established.

As inoculation into the skin of the Harderwijk dolphins was not allowed, cultures were sent to Dr. L. Ajello of the Communicable Disease Center at Atlanta, Georgia, for inoculation into dolphins in Florida. A serological approach of the etiology question was unsuccessful. The fungal cells in the dolphin skin did not stain specifically with anti-*Petriella setifera* fluorescent antibodies according to Mr. M. Reuver of the Laboratory for Microbiology of the University of Utrecht, whose help is here gratefully acknowledged.

The comparative cultural experiments with *Petriella* and *Petriellidium* strains were continued. The saline tolerance was investigated. Mycelial growth was not altered on Czapek agar containing 10% NaCl, but was somewhat retarded at a 4% concentration. A marked strain variability of this character was observed. The results were in good agreement with Kirk's report (Mycopath. Mycol. Appl. *33*: 65–75, 1967). On Czapek agar + 7% NaCl growth was either poor to very poor, or completely

inhibited; no growth occurring on 10% NaCl. All the strains grew profusely on Czapek agar containing 1%, 4%, 7%, or 10% sucrose. The wall of the young as well as the mature ascocarps of all strains in the ascus state was, in contrast to the young ascospores, neither metachromatic (in cresyl blue) nor dextrinoid.

A new *Epidermophyton* species (*E. stockdaleae* Prochacki & al.) received from the authors did not give rise to ascocarps in a mating experiment with the *Epidermophyton floccosum* strains maintained in the collection of the CBS.

From 7 soil samples out of a total of 18 collected in Eastern Flevoland keratinolytic fungi were isolated which belonged to *Trichophyton ajelloi*, *T. terrestre*, *Ctenomyces serratus* (first record since the beginning of the investigations) and an unknown *Anixiopsis* species, which was not recorded before and which will be studied at a later date. Soil samples (8) collected in Southern Flevoland did not contain keratinolytic species. Thermophilic and thermotolerant fungi and actinomycetes were isolated from most of the soil samples. The fungi were identified as *Aspergillus fumigatus* (from 9 localities), *Malbranchea pulchella* var. *sulphurea* (from 4 localities), *Chaetomium thermophile* (from one sandy-loamy field), *Thermoascus aurantiacus* (abundant at one locality) and *Mucor pusillus* (one isolate). One strain of the thermotolerant actinomycetes isolated from the soil collected in the Flevoland polders in May 1974 slightly inhibited the growth of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. None of the isolates obtained in 1975 was antibiotically active.

4. Division of Yeasts (Laboratory of Microbiology, Delft)

Head: Prof. T. O.Wikén

Drs. L. Rodrigues de Miranda

The investigation of the red basidiomycetous yeasts was continued. Mating tests were carried out on different media in order to find out which medium gave the best result. Phaff mentioned in "The Yeasts" good results on yeast-nitrogen-base agar plus l-arabinose, but this could not be confirmed. A mixture of Difco malt agar and cornmeal agar gave best development of true mycelium with clamps and chlamydospores (teliospores). All the 58 CBS-strains of *Rhodotorula rubra* were mixed four by four on this last-mentioned medium. No mating activity was observed. Most strains developed pseudomycelium after some time. The same mating tests have been carried out with all strains of the following species:

Rhodotorula araucariae (2 strains), *R. aurantiaca* (6 strains), *R. minuta* (4 strains), *R. minuta* var. *texensis* (12 strains), *R. pallida* (3 strains), *R. pillimanae* (5 strains). None of these strains showed mating activity.

Of the 8 strains of *R. graminis* two strains proved to be self-sporulating. Strain 5811, received from Goto, Yamanishi University, Japan, labelled *Sporobolomyces coprophilus* and described by Phaff as *R. graminis*, produces

thick-walled chlamydospores, mycelium with clamps and ballistospores on cornmeal-agar. As the assimilation of carbon-compounds fully agrees with the standard description of *Sporidiobolus ruinenii*, strain 5811 can be identified with this species. Strain 5624 also produces mycelium with clamp connections and teliospores as was reported in the progress report of 1974. Repeated efforts to grow ballistospores failed again. A determination of the GC content of the DNA-bases is necessary to find its proper taxonomic place. Strains 4648 and 4477 mixed together gave conjugation and true mycelium with clamp connections and thick-walled teliospores, which are larger than those of strain 5624. Ballistospores were not observed. A piece of mycelium with teliospores was isolated and cultivated on Difco-malt agar, but after some time the yeast phase overgrew the mycelium phase. A test was set up to find out whether the assimilation of the hydrocarbons octane-decane or hexadecane could be used as a criterion for the separation of species within the genus *Rhodotorula*. The results were unsatisfactory and the experiment was ceased.

A number of experiments were carried out together with Dr. T. Török (University of Horticulture, Budapest) with the mutagenic agent N-methyl-N-nitro-N-nitroso-guanidine on three different yeast species: *Candida albicans*, *Candida utilis*, and *Candida ingens*. Treatment of *C. albicans* and *C. utilis* did not result in spore formation, but in *Candida ingens* ascospores could be observed.

Drs. M. Th. Smith

During the study of the apiculate yeast genera *Hanseniaspora* and *Kloeckera*, Ogata's *Kloeckera* sp. No. 2201 (=CBS 6774) was obtained. In 1970 Ogata et al. published two papers on a methanol-assimilating yeast, tentatively identified as a *Kloeckera* species (J. Ferment. Technol. Osaka *48*: 389–396 & 470–477). According to Hazeu et al. (Arch. Mikrobiol. *87*: 185–188, 1972), however, the type strains of all *Kloeckera* species in the CBS culture collection do not assimilate methanol. Checking the cell form and the way of vegetative reproduction, it was found that CBS 6774 did not show bipolar budding and apiculate cells, features specific for *Hanseniaspora* and *Kloeckera*. Forming pseudomycelium and no ascospores and from the physiological characteristics, CBS 6774 could be identified as *Candida boidinii*, a species known to assimilate methanol.

The work on the genus *Trichosporon* has been started. *Trichosporon* is characterized by budding cells of various shapes, well developed or reduced pseudomycelium and the formation of true mycelium and arthroconidia. Two validly described species cannot be accepted. *Trichosporon hellenicum* Verona & Picci CBS 4099 does not produce arthroconidia, as has already been reported by Do Carmo-Sousa in "The Yeasts, A taxonomic study" (ed. J. Lodder, 2nd ed., North-Holland Publ. Co., Amsterdam). As it produces pseudomycelium, it can be placed in the genus *Candida*.

Ito et al. (Agr. Biol. Chem. *38*: 1597–1602, 1974) isolated a new radio-resistant yeast from rice and described this strain with a latin diagnosis as *Trichosporon oryzae* (CBS 6678). It does not form arthroconidia and therefore has to be placed in *Candida*; physiologically it resembles *C. edax*.

A yeast, labelled IMUR 2329 (=CBS 6721) and isolated from waste water, was validly described by De Queiroz as *Endomycopsis montevidensis* (Mycopath. Mycol. appl. *51*: 307–314, 1973). True mycelium and arthroconidia are formed. The ascospores reported by De Queiroz could not be found, even though several sporulation media were used. It is probably identical to *Trichosporon cutaneum*.

Two yeast cultures, isolated from soil near Pretoria, were received from J. P. van der Walt. One strain, CBS 6697, is a new nitrate-positive *Trichosporon* species and has been described by Van der Walt & Johannsen as *Trichosporon terrestre* (Antonie van Leeuwenhoek *41*: 361–365, 1975). The second strain, CBS 6699, could be identified as *Candida ciferrii*. Much time has been spent on mating experiments between five *C. ciferrii* strains (CBS 4856, 5165, 5166, 5295, 5646) and CBS 6699. These positive results will be published in 1976 in Antonie van Leeuwenhoek. Since *Candida chiropterorum* (CBS 6064), validly described by Grose & Marinkelle (Mycopath. Mycol. appl. *36*: 225–227, 1968), has the same physiological characteristics as *C. ciferrii*, this strain has also been crossed with all *C. ciferrii* cultures, including CBS 6699. Neither conjugation nor ascospores were observed.

D. Yarrow

The DNA base composition of some strains was determined. The yeasts were grown overnight in 4 l aerated medium (5% glucose, 0.25% peptone, 1% yeast extract), harvested by centrifuging, washed with saline-EDTA and with acetone, frozen and thawed several times, then lysed in saline-EDTA with 1% mercapto-ethanol and 2% sodium lauryl sulphate under chloroform vapour at 37° C. The DNA was purified with a combination of the techniques of Marmur (J. Mol. Biol. *3*: 208–218, 1961) and Kirby & al. (Biochem. J. *104*: 258–262, 1967). DNA was precipitated from cold solution with c. $^1/_3$ vol. of cold 2-ethoxyethanol instead of 1 vol. The melting point was usually determined 5 times for each DNA with the method of Marmur & Doty (J. Mol. Biol. *5*: 109–118, 1962) in a Gilford 2400-2 recording spectrophotometer at 260 nm. The cuvette chamber was heated at a rate of 0.25° C per minute by circulating anti-freeze through the thermospacers connected to a thermo-circulator. DNA of *Candida parapsilosis* CBS 604 (Tm 65.9° C) was included as standard in each determination. The mean Tms with standard deviation and percentages of guanine + cytosine (calculated from the formula %GC = (Tm-69.3)/0.41 are given below.

Organism	Tm	%GC
Saccharomyces dairensis		
CBS 1579	84.3 ± 0.13	36.6
Saccharomyces exiguus		
CBS 379	83.1 ± 0.09	33.7
CBS 1515	83.2 ± 0.11	33.9
CBS 2141	83.1 ± 0.05	33.7
CBS 5647	82.8 ± 0.08	32.9
CBS 6388	82.8 ± 0.15	32.9
CBS 6440	83.2 ± 0.11	33.9
Saccharomyces florentinus		
CBS 6773	86.7 ± 0.25	42.4
CBS 6802	86.5 ± 0.08	42.0
Saccharomyces microellipsodes		
CBS 427	86.1 ± 0.08	41.0
CBS 6142	85.9 ± 0.12	40.5
CBS 6641	86.1 ± 0.13	41.0
CBS 6762	85.9 ± 0.19	40.5
Nard. 3099	85.6 ± 0.10	39.8
Saccharomyces montanus		
CBS 4506	86.6 ± 0.18	42.2
CBS 6544	86.8 ± 0.16	42.7
CBS 6711	86.8 ± 0.06	42.7
CBS 6772	86.6 ± 0.11	42.2
Torulaspora delbrueckii race *florenzanii*		
CBS 2924	87.3 ± 0.14	43.9
CBS 2925	86.9 ± 0.13	42.9
CBS 5448	87.0 ± 0.15	43.2
Kluyveromyces sp.		
CBS 3019	83.4 ± 0.08	34.4
CBS 6750	83.5 ± 0.17	34.6

The species of the genus *Saccharomyces* sensu lato have been provisionally classified as follows:

1) *Saccharomyces* consisting of the species whose vegetative phase is predominantly diploid, conjugation occurring soon after germination of the ascospores. Five species can be recognized, namely *S. cerevisiae*, *S. kluyveri*, *S. exiguus*, *S. dairensis* and *S. servazzii*.

2) *Zygosaccharomyces* consisting of the species whose vegetative phase is predominantly haploid, conjugation occurring between individual cells just before ascosporulation, namely *Z. rouxii*, *Z. bailii*, *Z. bisporus*, *Z. cidri*, *Z. florentinus* (syn. *S. eupagycus*), *Z. microellipsodes*, *Z. montanus*, and *Z. mrakii*.

3) *Torulaspora* comprising species whose vegetative phase is haploid, ascus formation as a rule being initiated by somatogamous autogamy. Three species are retained, *T. delbrueckii* (syn. *S. rosei*, *S. fermentati*, *S. florenzanii*, *S. vafer*, *S. saitoanus*, *S. microellipsodes* var. *osmophilus*, *T. benedictae*, and *Debaryomyces nilssonii*), *T. inconspicuus*, and *T. pretoriensis*.

4) *S. telluris*, *S. transvaalensis*, and *S. unisporus* have to be placed in another, as yet undetermined, genus. Electron-microscopical examination of the ornamentation of the ascospore wall is necessary before a decision can be taken.

5) *S. kloeckerianus*, though its vegetative phase is usually diploid, has rough-walled ascospores and cannot be retained in the genus *Saccharomyces*. Neither can it be placed in *Torulaspora*, as somatogamous autogamy does not occur.

DNA studies will be continued to evaluate the validity of this provisional classification.

Of the newly described species examined this year *Candida hydrocarbofumarica* nom. nud. and *Candida inositophila* Nakase could be identified as *Candida steatolytica* Yarrow, and *Schizoblastosporion kobayashii* Soneda & Uchida as *Selenotila intestinalis* Krasilnikov.

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INSTITUTE FOR ECOLOGICAL RESEARCH - ARNHEM

Progress Report 1975

1. Function and organization of the institute

The institute was founded in 1954 by the Division of Sciences of the Netherlands Academy of Arts and Sciences. The function of the institute is to perform and to encourage terrestrial ecological research in a broad sense and to co-operate with other organizations engaged in such research.

The research projects of the institute are carried out by four departments, three of which are housed at the headquarters of the institute at Arnhem; the Department for Ecological Dune Research has its seat at Oostvoorne. Recently a new seat for experimental research was founded at Heteren near Arnhem. The field work is carried out in different parts of The Netherlands, (see Fig. 1).

The main objects of study of the institute are the properties of plants and animals in relation to their specific occurrence. In this respect special attention is paid to plants of dune grassland and to birds, particularly the Great Tit.

The research projects are performed by means of a multidisciplinary approach. For parts of these projects the institute closely collaborates with some departments of the universities. In addition the institute administrates the ringing of birds in The Netherlands. It is also the site of the provisional Euring Databank, in which all recoveries of birds ringed in Europe will be assembled.

The institute is supervised by a committee appointed by the Division of Sciences of the Academy. It is financed by the Government.

The addresses of the institute are:
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Department for Ecological Dune Research: "Weevers' Duin",
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D. van der Laan (Synecology)
S. R. Troelstra (Soil science)
M. J. Adriani (Guest worker)

3. Population ecology

3.1. INTRODUCTION (J. H. van Balen)

The Department of Population Ecology is concerned with the ecology of bird populations, with special attention to population dynamics.

Research on population dynamics includes the study of numbers, of fluctuations in numbers, and of the factors affecting the size of animal populations. The factors concerned are usually numerous, and they form a complex of interrelationships with the numbers and other population parameters of the species under study. For a good understanding of the ecological processes taking place in the population, research must be extended to other disciplines, e.g. ethology, physiology, and population genetics. This kind of approach has proved very productive, as the literature shows. The work on the population dynamics of the Red Grouse *Lagopus scoticus* by A. Watson and his associates may be mentioned as an example of an interdisciplinary study.

This approach requires the cooperation of a team of specialists from several disciplines. The comprehensiveness of the work involved (especially the field work) usually precludes research on more than one or a few similar species. For a number of years, the Great Tit *Parus major* has been the main species studied in our Department and it is our intention to continue this research. Not only has a considerable amount of knowledge on population processes been accumulated, facilitating further investigation of the mechanisms involved in population dynamics, but, in addition, a field of cooperative research has been opened, especially now that the behavioural aspects of population dynamics are included in the research program (see 3.2.2.).

The concentration of research on a single or a few related species may lead to a better insight into the population dynamics of the species concerned but does not garantuee the finding of phenomena that can be generalized to other species. Since the detection of generally valid phenomena should be considered a major goal of the study of population dynamics, this aspect should not be neglected. To this end, projects will be undertaken in

Fig. 1. Geographical position of the Institute for Ecological Research and its field work sites.

1. Headquarters at Arnhem.
2. National Park *De Hoge Veluwe*, main scene of the field work on the Great Tit.
3. Ermelo, main scene of the field work on the Collared Dove.
4. Vlieland, additional field work site of the Departments of Population Ecology and Bird Migration.
5. Westeinder Plassen, main scene of the field work on the Coot.
6. Oosterhout, additional field work on the Great Tit.
7. Liesbosch, additional field work on the Great Tit.
8–9. Zuidelijk and Oostelijk Flevoland, newly reclaimed IJsselmeer polders, scene of the field work of the Department of Distributional Ecology.
10. Dune system of Voorne, with the Biological Station Weevers' Duin, seat of the Department for Dune Research.
11. Dunes of Goeree, additional field work of the Department for Dune Research.
12. Heteren, seat for experimental Research.

which the existence of phenomena or processes found in the Great Tit will be studied in other species.

In this report some recent results of the research on the Great Tit and the Coot *Fulica atra* will be discussed.

3.2. POPULATION DYNAMICS OF THE GREAT TIT, *Parus major*

3.2.1. *Factors affecting the size of the breeding population* (J. H. van Balen)

As mentioned earlier (Van Balen 1975), the size of the breeding popula-

tion in our study area on the Hoge Veluwe was affected by the size of the beechmast crop and by the provision of extra food (seeds) during winter since 1967.

The effect of weather factors was investigated by comparing the census data of the breeding population, corrected for the influence of the beechmast crop, with monthly values for the mean air temperature, number of frost days, and number of days with snow. The values for the months of November to March were tested in different combinations. The results show that in the 1960–1966 period the size of the breeding population was not correlated with any of the weather factors, but the data for 1968–1974 yielded some striking correlations. The number of breeding pairs is larger the higher the mean air temperature in December-February and the lower the number of days with frost and snow. Thus, the size of the breeding population was primarily affected by the beechmast crop in the years before 1967, and by the winter temperatures and the provision of extra food in the years from 1967 onward.

Subsequently, changes in the composition of the breeding population were studied, and the annual survival of different categories of Great Tits was investigated in relation to the above-mentioned factors.

The composition of the breeding population has changed markedly in recent years. From 1966 onwards the proportion of autochtonous adults (i.e. those that nested locally in a previous year) has increased considerably, while the proportion of autochtonous yearlings (i.e. born locally the year before) has not changed, and the proportion of immigrants, mainly yearlings, has decreased strongly.

These changes in composition might be due to changes in the survival of the different categories of tits. This point was studied by relating the data on annual survival (from one breeding season to the next) with the beechmast data separately for the years with and without provision of extra food. The annual survival of the autochtonous adults proved to be positively correlated with the beechmast crop during the years from 1959 to 1965. From 1966 on, this relationship disappeared but the survival percentages improved. When the beechmast crop was poor, the percentage of survival under food provision was about twice as high as the survival without food provision (Fig. 2). With a good crop the survival percentages in the two situations do not differ. Hence, the autochtonous adults profit from the provision of extra food by increasing their survival in years with a poor beech crop.

The survival of the autochtonous yearlings (from fledging until their first breeding season) is also positively correlated with the size of the beech crop, but here the relationship applied to both situations, i.e. with and without food provision (Fig. 2). During years with a poor beechmast crop the annual survival was doubled by the provision of food, but not sufficiently to reach the high survival values found in years with a good beech crop.

The difference in the effect of food provision on the survival of adults and

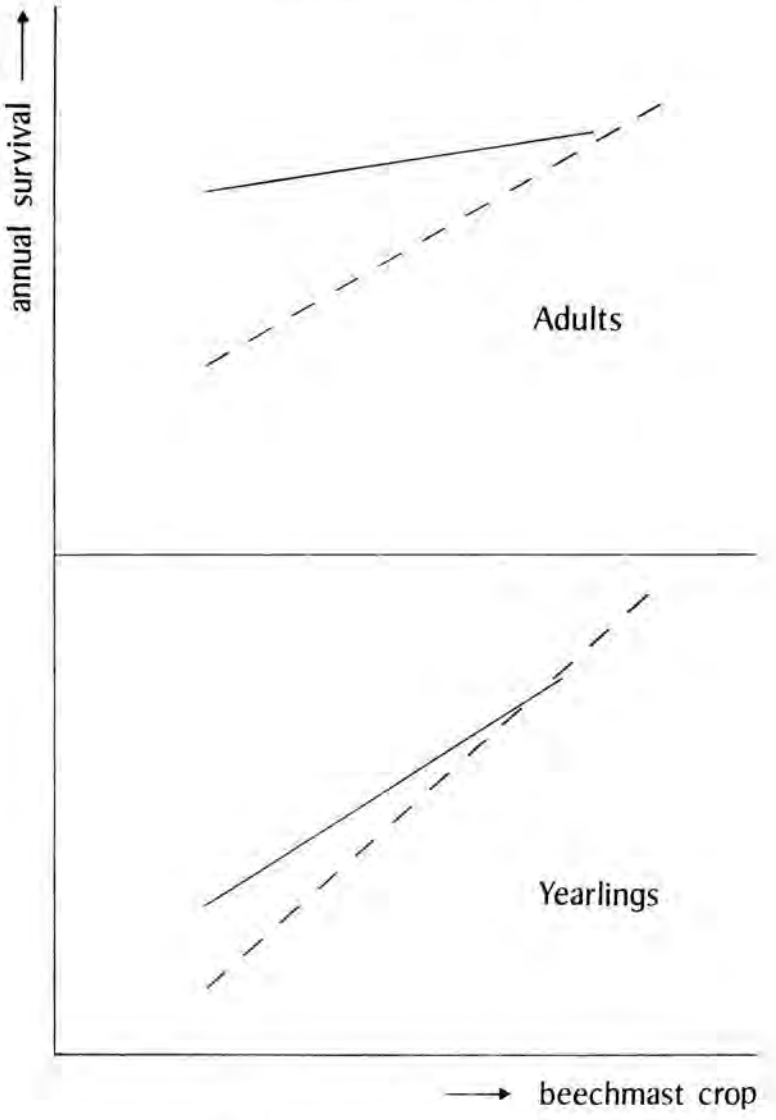

Fig. 2. Schematic picture of the relation between the size of the beechmast crop and the annual survival percentages in years with (————) and without (– – – – –) provision of extra food during the winter.

yearlings might be related to the uneven distribution of this type of food over the area. Beechmast is more evenly distributed, which enhances the yearlings' chances of feeding in spite of the presence of dominant adults.

Reference

Balen, J. H. van – Factors affecting the size of the breeding population. Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurk., 2e reeks, *66*, 80 (1975).

3.2.2. *Territorial behaviour and population dynamics* (P. J. Drent)

Several authors have suggested that certain aspects of behaviour, such as territoriality and social organization, have consequences for the determination of the size of bird populations. With respect to the Great Tit, divergent views are held on the limitation of numbers of breeding birds by territorial behaviour (e.g. Kluyver & Tinbergen 1953; Lack 1966), but a thorough investigation of this crucial problem has yet to be made.

The basic idea is that territorial behaviour excludes some individuals from establishing a territory in the study area, thereby either preventing them from reproducing or forcing them to reproduce in less favourable habitats, in which both breeding success and adult survival are lower. To test this idea, a thorough study of territorial settlement, including the causation and development of territorial behaviour and the adaptive significance of the territory, is needed. This study should be combined with an investigation of the ecological factors affecting reproduction and survival.

A first approach to this problem was made between 1968 and 1973 at the University of Groningen. The field work was carried out in Vosbergen, near Eelde. In January 1975 a new project was started in the Hoge Veluwe area near Arnhem, as a continuation and extension of the work done at Vosbergen.

The two main questions to be answered are:

1. Does the complex relationship between behaviour and population dynamics found in one habitat also apply to other conditions, and if not, what is the cause of any differences?
2. What is the nature of the relationship between feeding conditions, dispersal, territoriality, and size of the breeding population? This aspect, which will be studied experimentally, fits in with the descriptive and numerical study reported earlier (see under 3.2.1.).

The intensive study area is a rectangle of about 75 hectare, part of the department's main study area in the southern part of the Hoge Veluwe region, and is characterized by relatively large monocultures of oak, birch, Scots pine, and larch, with some beeches locally. Since 1966, extra food has been provided in the winter (September-March) at two sites in the intensive study area and at four sites in the remaining area.

In the first two seasons of the study (1974/1975 and 1975/1976) extra food was provided as before; later on, food provision will be stopped for two seasons. The first season was one with a rich crop of beechmast, but during the second there was no crop at all. There are good reasons to expect a similar succession of rich and poor crops for the next two years (1976/1977 and 1977/1978), which will enable us to compare the influence of human food provision and of the beechmast crop independently.

The size of the Great Tit population and the identity of the individuals present in the intensive study area is assessed by various methods: nestbox inspections and capture of the adults in the breeding season, weekly captures at feeding and drinking sites, nocturnal inspection of roosting

boxes, and identification of colour-ringed individuals in the field. The selection of foraging areas is studied by recording the foraging locations and numbers of tits along a fixed route twice a week. The intensity of territorial behaviour and the identity of territorial birds is studied by presenting a playback of song, combined with a dummy, at regular intervals along a route covered once a week. Incidental observations are made to obtain a complete picture of the territorial system and changes in it throughout the year.

Territorial behaviour in Great Tits is usually accompanied by song or by distinctive calls, such as "tinking" or "churring". All three types of sound show a strong seasonal fluctuation in frequency of use, with a spring peak (February-April) in which all three occur frequently, and a autumn peak (mid-August-October) in which mainly "tinking" and "churring" are used. This shows that song alone does not give a reliable picture of territorial activity throughout the annual cycle.

The males present in early February could be classified according to age and place of birth or breeding. Territorial ownership increased with increasing age and duration of stay in the study area (Table 1). Local survival was not, however, affected by these two factors. In contrast, the local survival of late immigrants (first capture after February 1st) was appreciably lower, viz. 20% (n = 15). The local survival of males with or without a territory on February 1st did not differ (72% and 71%, respectively). However, within the group of territorial males local survival decreased with age (Table 2). The observations suggest that intraspecific competition played a role in this phenomenon.

Table. 1. Territorial ownership and local survival in relation to age and history of the males

Age/history	Number present on 1 Feb.	% territory-owners	% present in breeding season
Yearlings, born locally (early)	16	87.5	75.0
Yearlings, born locally (late)	18	38.9	77.8
Yearlings, immigrants before 1 Feb.	10	30.0	70.0
Older autochtonous birds	34	97.1	67.6

Table 2. Local survival of territorial males in relation to age

Age	Number present on 1 Feb.	% present in breeding season
Yearlings	24	79.2
Second-year males	19	73.7
Third-year and older males	14	57.1

In the breeding season 83 males were present, 19 of which were non-territorial. Due to a shortage of females, several males were unpaired, viz. 2 territorial and 5 non-territorial males (3% and 26%, respectively, of the number present). Thus of the 76 pairs present, 14 had no territory. All non-territorial pairs, however, nested as subdominant birds within the territory of other males. The same phenomenon was found in the Vosbergen study.

When the males are classified according to age, territorial status, and date of territorial settlement, and when several aspects of the reproduction are compared, few differences are apparent. The date of laying, clutch size, and breeding success did not differ among territorial males of different age and history. The main difference was the late onset of egg-laying in the non-territorial group (6 days later than the territorial pairs, and 12 days later than the pairs in whose territory they nested). Moreover, the local survival of the young reared by these pairs was very low compared with that of the rest of the fledglings. This was probably not due to a high mortality shortly after fledging, since the weights of the young did not differ, but rather to dispersal beyond the boundaries of the study area, possibly to the area where the parents had once lived.

There were marked seasonal fluctuations in the use of different tree species for feeding. From January to March, about 60% of the foraging flocks were observed in and under beeches and at the artificial feeding sites. Birches formed the preferred species for foraging in April and May, but feeding was mostly confined to the herbage under the trees. Starting in June oaks were preferred as feeding sites, and this preference persisted until August, when the tits turned to birches and Scots pines. Due to crop failure beeches were not visited in the autumn of 1975.

Attempts to establish territories took place predominantly in areas favoured for feeding. This applies to attempts in February-March, which mainly occurred near beeches or artificial feeding sites, and to attempts in late summer, which often occurred in birch plots.

The influence of beechmast and of artificial food provision on Great Tit populations is not expressed as an effect on the settling of juvenile males in early autumn, but should be regarded as affecting survival, dispersal, and the replacement of lost territorial birds during winter and spring.

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3.2.3. *Experiments on the regulation of numbers in the Vlieland population* (J. H. van Balen and H. M. van Eck)

The analysis of the results of the experiments on the effect of removing part of the breeding population was continued. The removal of part of the breeding population in the summer proved to improve the local survival of

the remaining adults. The effect is larger for birds in their first breeding season than for older birds. Juveniles profit appreciably from the removal of breeding adults, especially of birds in their first breeding season. The results indicate that with increasing age the tits secure an increasingly stronger dominance position, which makes them less dependent on the presence of conspecifics.

3.2.4. *Eco-physiological aspects of reproduction in Great Tits*
(J. A. L. Mertens)

The range of temperature tolerance of Great Tit broods.

In previous years the influence of brood size and ambient temperature on the rate of heat loss in Great Tit broods was studied (Mertens 1971 and 1973). A model in which the physical properties of nests and nestboxes are operative was designed for prediction of the rate of heat loss of broods of different sizes in response to different ambient temperatures.

The model has been improved considerably in recent years. A major improvement was attained by extending the model by the incorporation of heat transport mechanisms due to water evaporation and water vapour diffusion through the nest. Another improvement was made by adding allometric equations concerning the upper and lower limits of metabolic heat production by Great Tit nestlings of different ages as well as the evaporative heat loss of the nestlings. The model which can be now used to calculate the range of temperature tolerance of Great Tit broods of different sizes and ages and in nestboxes of different dimensions and physical properties, has been described in two papers which will be published soon.

The allometric equations which describe the minimum and the maximum metabolic heat production of the nestlings in this model were derived from the results of experiments on Great Tit nestlings performed over several years. The equation which relates the minimum (basal) metabolic rate to the nestling body weight deserves special consideration, and is discussed in the next section of this report.

The basal metabolic rate of Great Tit nestlings

The results of experiments on nestling Great Tits show that the basal metabolic rate q_{bmr} can be described as a function of the body weight M_b (in kg) according to the equation:

$$q_{bmr} = 56.1\ M_b^{1.278}\ \text{Watt} \qquad \text{(eq. 1)}$$

which differs considerably from the equation describing the basal metabolic rate of adult passerine birds:

$$q_{bmr} = 6.25\ M_b^{0.724}\ \text{Watt} \qquad \text{(eq. 2)}$$

(modified after Lasiewski & Dawson 1967).

According to calculations with these equations, the basal metabolic rate over the whole range of nestling body weights (from 1 to 18 grams) is always lower than the basal metabolic rate of adult passerine birds of comparable body weight. Moreover, it can be seen from the equations that the basal

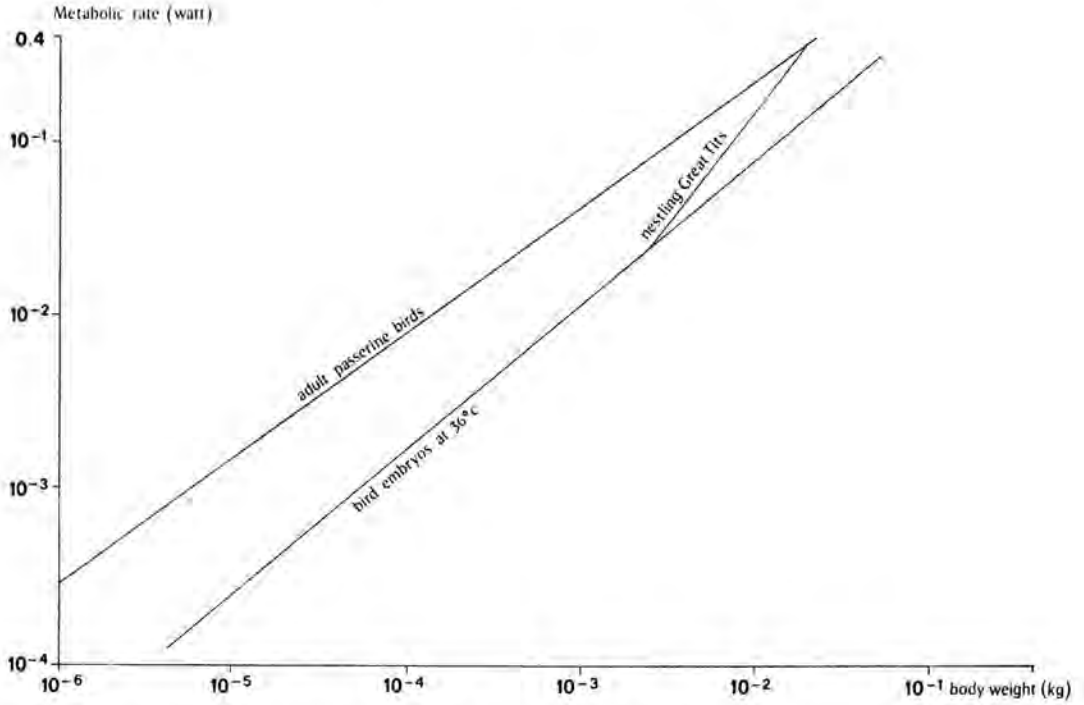

Fig. 3. Metabolic rate levels of bird embryos, adult passerine birds and nestling Great Tits as a function of their body weight.

metabolic rate per gram body weight decreases with increasing body weight when different species of adult passerines are considered, but increases when the weight-specific basal metabolic rate of Great Tit nestlings differing in body weight is taken into consideration. A similar trend was also found for nestlings of other species, and some authors regard this as an adaptation serving to economize fats and proteins in order to promote a rapid growth rate. It seems more plausible, however, to consider the low metabolic rate during the nestling period as a characteristic inherited from the early ancestors, the reptiles. It is interesting to note that the metabolic rate of bird embryos during incubation, which normally occurs at about 36°C, is the same or only slightly higher than the metabolic rate of reptiles at this temperature (Mertens, in prep.). This of course does not exclude the possibility that birds profit from the low metabolic rate in the early stages of development, saving energy for growth, but this should not be considered as an adaptation; but merely as a gift from their ancestors. Fig. 3 shows how the metabolic-rate levels of bird embryos, adult passerines, and Great Tit nestlings are related to each other.

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Mertens, J. A. L. – A model for the prediction of heat loss of Great Tit broods. Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurk., 2e Reeks, *61*, 86–90 (1973).

3.2.5. *The breeding of Great Tits in natural nest sites* (J. H. van Balen and K. Booy)

In population studies on tits artificial nest sites (nesting boxes) are commonly used. Evidence has accumulated indicating that clutch size is dependent on (among other factors) the size of the nesting box, and that nestling survival depends on certain properties of the nest site related to the transport of heat and moisture. Since natural nest sites differ from nesting boxes in a number of important features, the breeding success in the two types of nest site may also differ considerably. The lack of research on the breeding biology of hole-nesting birds in natural nest sites has left an obvious gap in our knowledge. In 1975 a start was made on filling this gap.

The study was performed in a situation in which tree-holes were concentrated in a small part of the available area, viz. where rows of medium to old deciduous trees (beeches, oaks, birches) were situated in large complexes of coniferous or young deciduous plantations. An inventory of all inhabitable holes showed that 102 of the 118 available holes were occupied. The Starling *Sturnus vulgaris* was the main inhabitant (59% of all clutches), with the Great Tit (18%) taking second place. Most of the Great Tit nests were found in long narrow clefts (in beeches) and in other narrow holes unsuitable for Starlings.

The breeding success of the Great Tit was very low: only 40% of all occupied nests resulted in at least one fledged young. The mean clutch size was 7.1, the mean number of hatched young 5.3, and the mean number of fledged young (in successful broods) 4.6. These results are poor compared with the usual breeding success in nesting boxes, but in 1975 the breeding success in the neighbouring Hoge Veluwe area was equally poor. This study should certainly be continued.

Several measurements were made from which the bottom-surface and the volume of the cavities could be estimated. Both varied enormously, the bottom-surface from 24 to 300 cm^2 (mean 137 cm^2) and the volume from 0.7 to 8.8. dm^3 (mean 3.3 dm^3) ($n = 29$).

The size of the bottom-surface and the volume were correlated with several parameters of the Great Tit's breeding biology. Significant positive correlations were found between bottom-surface and clutch size and between bottom-surface and number of fledged young. The correlation between bottom-surface and egg – and nestling – mortality proved to be negative. All correlations are consistent with the hypothesis that large holes are preferred to small holes, and that in large holes clutches are larger and mortality is lower than in small holes.

The preliminary results suggest that Great Tits often nest in cavities of smaller size than those in which breeding success is maximal. This is presumably due to competition for nest sites with Starlings.

3.2.6. *Parasitism by Protocalliphora spp.* (C. W. Eshuis-van der Voet and E. Houwink)

The observations done in Switzerland were concerned with the biology of *Protocalliphora*. Some results will also be reported of a study done in the Hoge Veluwe region by J. den Boer-Hazewinkel (in 1974), on the relationship between the Great Tit and *Protocalliphora*.

A number of nestboxes with Great Tit's clutches were examined in both the Hoge Veluwe and Bischofszell (Switzerland), but no eggs of the fly were found. The egg stage probably lasts about 30 hours.

Observations were done on the timing of the larval moults, but no firm conclusions could be reached. The larvae usually take two meals of blood daily from the nestling tits. Sucking of blood seems to occur both through the skin and from the quills. A number of arthopod predators of larvae were found, notably *Gnathoncus buyssoni* Auzat and *Haploglossa pulla* Gylh. The risk of this predation was highest for the youngest larvae.

The relationship between pupal emergence and relative humidity was investigated. The percentage emerging proved to be high (90–100%) at all humidities (34–100%).

Attempts to observe or capture imagines in the wild failed. Nesting boxes at the edge of the woods were infected more heavily than those inside the woods.

Observations in the Hoge Veluwe area showed that the number of infections is directly related to the number of available tit broods, except at the end of the breeding season, the last broods being more heavily infected. Infections were highest in broods aged 7–8 days. The smaller number of infections before this stage may be due to disturbance of the flies by the brooding females, and after this stage by the increased mobility of the nestlings.

In nests artificially infected by the observer the larvae usually disappeared in the course of 1 to 3 days, probably due to the activities of the parent tits. This holds especially for flimsy nests.

3.3 ECOLOGY OF THE COOT, *Fulica atra* (J. Visser)

From 1965 onward, the composition of the Coot population in two adjacent areas, the Westeinderplassen and the Ringvaart Haarlemmermeer, has been studied by regular captures of ringed birds throughout the year. The age, sex, and status of the birds were determined from ringing and measurements. Measurements and observations were also collected on moult, wing length, body weight, frontal shield, and leg colour. As far as possible, the influence of the environment and of the composition of the

population on these parameters is also being studied. Papers dealing with wing length and body weight of fullgrown birds are in preparation.

The most important part of the study, however, concerns the reproductive cycle of the Coot in different years and habitats, with special attention to the internal and external factors affecting population density, the date of the first egg, clutch size, weight and composition of the eggs, brood size, and growth and survival of the chicks. So far, only results concerning the growth (body weight and wing length) of young Coots have been published (Visser 1974). Several internal and external factors were found to affect the growth of young Coots.

It is likely that in particular the female parent plays an important role in the reproductive cycle, because the date of the first egg, the clutch size, the weight and composition of the eggs are probably correlated with the age and condition of the female. We intend to study this aspect in a more experimental way in a pond in Heteren. A short survey of some findings related to the reproductive cycle is given here.

Table 3 shows some data on the size of first clutches in different areas. For the Westeinderplassen area a distinction can be made between the clutch size of yearling and older females. It is clear from the 95% confidence limits that the difference between the two groups is significant. There is also a marked difference in mean clutch size between the Molenpolder and the other areas.

Table 3. Size of first clutches in different areas

area	mean clutch size	sample size	standard diviation	95% confidence limits
Molenpolder	5.48	97	1.32	5.21–5.75
Ringvaart	7.74	155	1.66	7.30–8.18
Westeinderplassen (a)	6.89	76	1.61	6.52–7.26
Westeinderplassen (b)	7.69	246	1.56	7.49–7.89

(a) = clutch size of yearling females
(b) = clutch size of older females

The differences in clutch size between age classes and between populations led us to study the weight and composition of the eggs. For this purpose large numbers of eggs (both fresh and incubated) were weighed, and samples were analysed. The preliminary results show a significant difference in egg weight (about 2 g) between the populations of the Westeinderplassen and the Molenpolder. Furthermore, eggs of yearling females are on average about 3 g lighter than those of older females. There are also indications that the egg weight is highest early in the season. These differences hold for fresh eggs as well as for well-incubated eggs. Therefore,

it seems probable that several components of the eggs that may be important for the survival of young chicks, are present in larger quantities in the eggs of older females than in those of yearling females. The same reasoning of course holds for the comparison of areas or parts of the season with a different mean egg weight.

So far, there are no indications that the composition of the eggs (percentage fat, protein, ash, and caloric value) is correlated with the age of the female or differs from area to area. The only differences found concerned the composition in the course of the breeding season. There is not only a significant decrease in the percentage dry matter, but also in the amount of fat as a percentage of dry matter. These two trends result in a considerable decrease in the total weight of fat per egg in the course of the season.

Summarized, we found the following pattern in early clutches (compared with late clutches):

1. more eggs (the clutch size decreases in the course of the season),
2. higher egg weight,
3. higher percentage dry matter, and
4. higher percentage of fat.

From the foregoing it seems evident that it is advantageous for a Coot to start egg laying as early as possible. In this connection it is clear that the older females are more successful in raising chicks than younger ones, because the older females start breeding earlier in the season than the younger females.

Reference

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4. Bird migration

4.1. SPONTANEOUS ACTIVITY OF CHAFFINCHES IN THE KRAMER CAGE

(A. C. Perdeck and C. Clason)

The study on the spontaneous migration activity of Chaffinches in the Kramer cage was continued. In the previous report (Perdeck 1975) an experiment was described in which the direction choice is compared between two groups: one (the normal group) kept under the artificial daylength of 60° northern latitude and the other (the season-shifted group) under the same conditions but set back six months as regards the daylength rhythm. When tested in the autumn of 1974, the latter group was significantly directed in the expected NE direction, but the normal group was not directed significantly, although the mean direction was also NE. During the spring of 1975, there was again no difference in direction between the two

groups. Application of Mardia's uniform-scores test led to the following conclusions:

1. The directions of 7 season-shifted and 7 normal birds, each tested one day during the autumn (25 August–25 October), did not differ significantly ($P > 0.10$).
2. The same holds for 8 season-shifted and 10 normal birds each tested one day in the spring (19 Feb.–21 April).
3. If only birds tested in both the autumn and spring (3 season-shifted and 5 normal) are selected, points 1 and 2 are confirmed.
4. When all autumn tests are combined, a significant choice of direction with a mean at 61° (14 individuals, $P < 0.005$) is found. All spring tests combined have a mean direction of 89° (18 individuals, $P < 0.001$). There is no significant difference between autumn and spring directions ($P > 0.10$).

In general, it must be concluded that the birds showed a preference for the NE and E sectors, irrespective of season and treatment. But in tests without the artificial sun (i.e., with only diffuse light) no significant direction choice could be detected (12 individuals in the autumn, 14 in the spring, each tested one day, $P > 0.10$ in both seasons). The easterly directions must therefore be induced in some way by the artificial sun. Since this artificial sun burned only during the first six hours of the day, with an azimuth direction changing from E to SSE, and taking into account the rather large scatter of the directions, simple phototaxis to the artificial sun cannot be excluded. However, an appreciable number of birds did not follow the course of the artificial sun but had a rather fixed direction throughout the day, and this is true for 8 birds tested in both the spring and autumn. It is conceivable that the articificial sun, though moving, facilitated the finding of some unknown fixed cue. The other alternative, a real sun compass orientation, is also possible, but then it is strange that the same direction persisted throughout the year.

Efforts were also made to change the direction choice by varying the temperature and magnetic field but without results. The tendency to select easterly directions persisted. We suspect that either there is something wrong with out experimental set-up or that under the experimental treatment, the birds are unable to display the orientation behaviour we want to study. Therefore, we decided to stop this kind of experiments.

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4.2. EXPERIMENTS ON THE DISCRIMINATION BY THE STARLING BETWEEN GEOGRAPHICAL LOCATIONS (A. J. Cavé, C. Bol and G. Speek)

The purpose and design of the experiments on the discrimination between geographical locations by the Starling are discussed in detail in the 1970 Progress Report (Cavé 1971). In these experiments the birds were trained to obtain food by jumping on one of two perches; at The Hague (Monster) by jumping on a black perch and at Münster (Gimbte) on a white one. It was concluded in previous reports that the birds are able to distinguish between these two localities at the place of training, both with the sun visible and under total overcast. It was further concluded that they are still capable of distinguishing the correct location in a different landscape at a short distance from the training places, when the sun is visible.

A thorough statistical analysis showed that there was considerable difference between the behaviour of the birds. Since two of the six birds seem to be able to distinguish the two locations in an unknown landscape under total overcast, we suspect that these two birds used cues other than the sun for recognising the two places. It cannot be excluded, that these other cues concern systematic differences in the conditions during the journey from Arnhem to The Hague or Münster or at the training places. Although it is attempted to minimize these differences, it is impossible to exclude all of them.

Since, in general, the results of these experiments are disappointing in relation to the enormous amount of effort they require, this study will not be continued.

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5. Distribution ecology

5.1. EFFECT OF CHANGES IN WATER TABLE ON THE VEGETATION DEVELOPMENT OF REEDBEDS IN THE ZUID-FLEVOLAND AREA (K. Reinink and J. van der Toorn)

Introduction

After reclamation of an IJsselmeer polder, rapid vegetation development results in a more or less closed reed stand (Feekes & Bakker 1954). This process can be accelerated by artificial sowing of reed, giving a closed stand within three years. After some years the growth of the reed diminishes, especially when drainage is applied. A number of simultaneous changes in the environment of reed could induce this decline, viz. (1) drying-up of

the soil; (2) a decrease in the mineral nitrogen content of the soil (Van Schreven 1965), and (3) an infestation of the reed by larvae of noctuid moths. To investigate each of these factors, the following experiment was set up in 1971 in the Zuid-Flevoland polder (reclaimed in 1968). A homogeneous reed field (surface 50 ha) was divided into 3 parcels with different water regimes. Each parcel was subdivided into two parts, one of which was burnt off each year and the other left intact, giving 6 treatments in all (Table 4). Burning (in the period March-early April) was applied as an effective method to control moth larvae. The preliminary results of this experiment show that the decline in reed growth is mainly caused by moth larvae: in the dry, undisturbed treatment those of *Rhizedra lutosa*, but initially also of *Archanara geminipuncta*, in the wet undisturbed treatment mainly the latter species (Mook & Van der Toorn 1974 and 1975). Since this experiment was also performed to investigate the effect of declining reed growth on plant succession, vegetation development was studied in the undisturbed treatments. This was done by two methods, i.e., in (1) permanent plots and (2) aerial photographs (false colour). Because the analysis of the photographs has not been completed yet, only preliminary results based on the permanent plots will be given here.

Table 4. Treatments applied in the experimental reed field in the Zuid-Flevoland polder

	Fluctuations in water level (cm)		
1 April–1 Nov.	+10/+20	−10/−90	−30/−90
1 Nov.–1 April	0/+10	0/−30	−20/−30
	Wet burned	Swampy burned	Dry burned
	Wet undisturbed	Swampy undisturbed	Dry undisturbed

Methods

A number of fixed transects, subdivided into small plots measuring one square meter, were used. The vegetation development in each plot was described yearly. At the beginning of the experiment a number of aggregates of *Epilobium hirsutum* occurred, this species being virtually the sole companion of the reed. To follow the succession within and outside these aggregates, 4 transects, each consisting of about 8 plots, were made in 1973 through separate aggregates in the objects wet and dry undisturbed. The same was done in 1974 for aggregates of *Cirsium arvense* and *Solanum dulcamara*. In this case long transects were used (in all three undisturbed objects) to permit investigation of plant succession outside the mentioned aggregates in a more general way as well. Those made in 1973 are referred to as "short" transects, the 1974 group as "long" transects. In all plots the observations (made in September/October, and starting in 1974 also in June) concerned the density, coverage, phenology, and height (all esti-

Table 5. Presence and coverage of plant species (except *Phragmites australis*) in the short transects, 1972–1975.

Presence is expressed as percentage plots containing relevant species (N = number of plots per object), coverage as mean percentage per plot.
M* = Perennials present at start of experiment
M = Other perennials
H = Hapaxants

		Presence								Coverage							
		1972		1973		1974		1975		1972		1973		1974		1975	
		–	X	–	X	VI	IX	VI	IX	–	X	–	X	VI	IX	VI	IX
DRY UNDISTURBED (N=33)																	
Epilobium hirsutum	M*		36								7						
Scirpus maritimus	M*		24		24	21	21	30	30		<1		<1	<1	<1	<1	<1
Cirsium arvense	M						6	24	27						1	4	6
Solanum dulcamara	M				9	12	15	18	18				1	3	9	9	10
Ranunculus acris	M				3								<1				
Polygonum lapathifolium	H				33	30	30	36	33				4	2	7	2	3
Sonchus asper	H				9	15	3	21					<1	<1	<1	<1	
Atriplex hastata	H				45	73	76	76	82				7	12	23	5	16
WET UNDISTURBED (N=36)																	
Epilobium hirsutum	M*		36								10						
Scirpus maritimus	M*		11		11	11	11				<1		<1	<1	<1		
Typha latifolia	M*		6		6	6	6	6	6		<1		<1	<1	<1	<1	<1
Ranunculus sceleratus	H							6								<1	

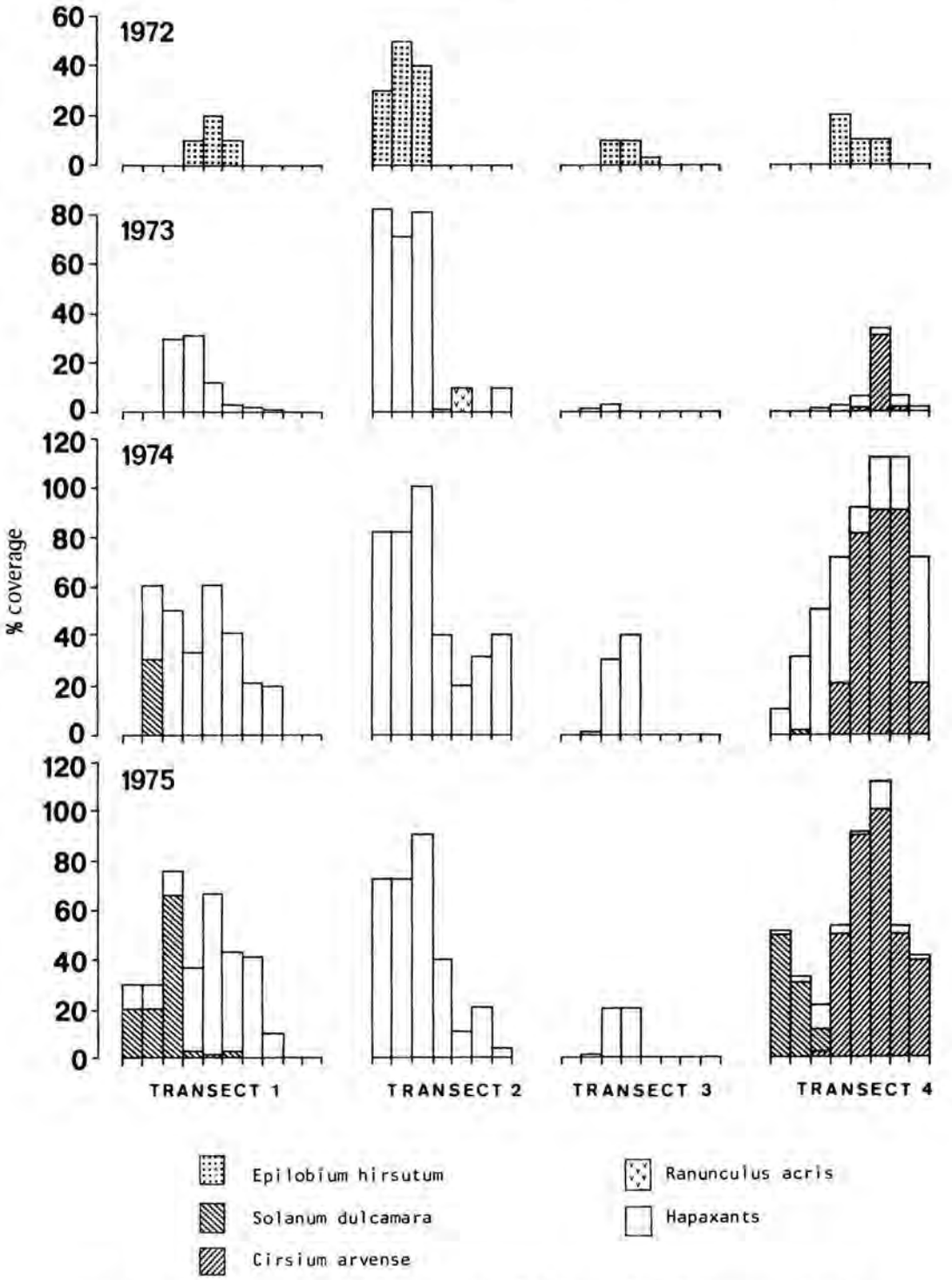

Fig. 4. Coverage per 1 m² plot in 4 transects of the dry undisturbed treatment in the period 1972–1975.

mated according to the scale of Barkman, Doing & Segal 1964) of the plant species present. For 1972, data for *E. hirsutum* were estimated from dead parts in 1973.

Results

Table 5 shows the pattern of plant development in the short transects for the period 1972–1975. Plant species are classified as perennials and hapaxants. The latter only flower once before they die and are mostly annual and biennial species. Of the perennials already present in 1972, *E. hirsutum* disappeared suddenly in 1973. This loss was caused by a heavy infestation by *Haltica lythri (Coleoptera, Chrysomelidae)*, which killed the young shoots in the spring of 1973, after which no regrowth occurred.

Table 6. Presence of plant species (except *Phragmites australis*) in the undisturbed treatments 1974–1975 (long transects).

Presence expressed as percentage plots containing relevant species.
N = total number of plots per object

		Dry (N=162)		Swampy (N=149)		Wet (N=88)	
		1974	1975	1974	1975	1974	1975
Scirpus maritimus	M*	8	8	8	12	6	3
Phalaris arundinacea	M*			3	3	6	6
Iris pseudacoris	M*			1	1		
Typha latifolia	M*					3	3
Salix triandra	M*			1	1		
Cirsium arvense	M	31	56	23	31		
Solanum dulcamara	M	13	17	18	23		
Epilobium hirsutum	M	5	4		2		
Epilobium spec.	M	1	2		1		
Poa spec.	M		3		2		
Tussilago farfara	M		1				
Lolium spec.	M		1				
Sambucus nigra	M			1	1		
Taraxacum spec.	M				1		
Oenanthe aquatica	M						1
Atriplex hastata	H	28	31	7	11		
Sonchus asper	H	14	24	1	5		2
Polygonum lapathifolium	H	17	23	1	4		1
Cirsium vulgare	H	15	13	7	9		
Ranunculus sceleratus	H	9	4	1	3		10
Chenopodium rubrum	H	5	3				
Erigeron canadense	H	3	1				
Rumex maritimus	H	3	3	1			
Matricaria inodora	H	2	1				
Stellaria media	H	1	1				
Moss species					6		
Total number of species		15	18	13	17	3	7

For explanation of M*, M, and H, see Table 5.

In the dry treatment a number of new species became established in 1973, the most prominent being the annuals *Atriplex hastata* and *Polygonum lapathifolium*. These species showed an increase in 1974, followed by a decrease in 1975. During these two years the perennials *C. arvense* and *S. dulcamara* became more important. There are rather large differences between presence and coverage for some species. *Scirpus maritimus* is a "relict" species, still occurring in many plots (high presence) but with a low coverage. *S. dulcamara*, on the contrary, is an invading species occurring in a limited number of plots (low presence) but with a relatively high coverage. Data on the coverage per plot are given for each of the perennial species in Fig. 4 (except *S. maritimus*, because of its low coverage). The hapaxanthous species are combined. The establishment of new species in 1973 was clearly more or less restricted to the old *Epilobium* aggregates, but in later years was also seen elsewhere (e.g. *C. arvense* in 1974 and hapaxants in 1974 and 1975). As Table 5 shows, the species composition in the wet treatment remained virtually unchanged except for the disappearance of *Epilobium* in 1973.

Table 6 shows the occurrence of species in the long transects. There is a considerable difference in species composition between the dry and wet treatments. The difference between the "dry" and "swampy" objects is much less pronounced (this is in agreement with the relatively small difference in water regime; see Table 4). However, in the dry treatment A. *hastata*, *Sonchus asper*, and *P. lapathifolium* occur more frequently. In all three treatments the number of species increased from 1974 to 1975.

Table 7. Mean above-ground biomass of reed in Augustus and September, according to year (dry weight in g per m^2)

Treatment	1971	1972	1973	1974	1975
Dry undisturbed	1,091	767	552	667	757
Dry burned	1,004	1,250	1,289	1,334	1,306
Wet undisturbed	1,128	937	884	1,318	1,268
Wet burned	1,039	1,495	1,340	1,412	1,774

Discussion

The difference in vegetation development between the dry and wet treatments can possibly be explained by two factors, viz. (1) differences in the growth of reed in dependence of the degree of infestation by noctuid larvae, and (2) difference in water level. In the dry treatment reed performance was distinctly inferior during the entire period of the study (Table 7). The difference in above-ground biomass between the two objects is most pronounced for 1974–1975, when the reed in the wet treatment showed a recovery. In addition to the better reed growth, the high water level may

have prevented the establishment of new species in the wet object. An indication for this possibility is provided by the finding that –in contrast with the dry treatment– new species did not invade the old *Epilobium* aggregates in 1973. In 1975, some new species occurred in the wet treatment. Their germination was perhaps made possible by the thick layer of reed litter, which had increased considerably since 1973. The new species noted for 1975 were all present as juvenile plants which disappeared later in the summer.

The rapid vegetation development in the dry and swampy treatments was due to both declining reed growth and a high mineral content of the soil. The fact that the decline in reed growth was caused by a sudden drop of the water level, raises the question of whether the observed vegetation development resembles that occurring in more natural situations. In the natural succession other species, adapted to higher water levels, will dominate. On the other hand, the processes leading to a decline in reed growth would be essentially the same, because *R. lutosa*, the main parasite of reed in dry habitats, also occurs under more swampy conditions.

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5.2. GERMINATION EXPERIMENTS WITH *Cotula coronopifolia* AND *Ranunculus sceleratus* (J. van der Toorn and H. J. ten Hove)

Introduction

Cotula coronopifolia, a plant species belonging to the *Compositae*, was found in Zuid-Flevoland (an IJsselmeer polder reclaimed in 1968) in 1972. Since then, the species has increased there considerably, both in number and area. *C. coronopifolia* was introduced into Europe in 1739 (first found near Emden, West Germany), and probably originated from South Africa (Hegi 1928). Within about 100 years it spread from Emden to Harboøre (in the northern part of Denmark), both along the coast and in inland places. Thereafter, *C. coronopifolia* was further dispersed to other places along the west coast of Europe (from Spain to the southern part of Norway and Sweden). After about 1850 the species showed a decline in NW. Germany and Denmark, and maintained itself only in some coastal areas (chiefly at

the mouth of the rivers Weser and Elbe). Along the English and Danish coasts the species has an irregular and ephemeral occurrence.

These data give rise to the following questions: how could the subtropical species *C. coronopifolia* establish and maintain itself under such different climatic conditions, and what is the explanation of its spread and decline in Europe? To answer these questions the ecology of *C. coronopifolia* was studied in relation to the climatically well-adapted species *Ranunculus sceleratus*. Both species are comparable in life cycle and habitat, since they are annual pioneer species of bare, wet and nutrient-rich mud in Zuid-Flevoland. Earlier experiments have shown that both have a high seed output, produced mainly during September and October. The seeds remain on the inundated soil during the winter. In the experiments described here, the effect of submergence on the germination behaviour of the seeds was investigated.

Materials and methods

Freshly harvested seeds of both species were divided into two lots, which were stored as follows:

(A) in submerged soil under field conditions from 17–9–1974 to 21–3–1975 (referred to as soil storage), and

(B) under dry conditions at +5°C (referred to as cold storage).

Germination under 6 different temperature regimes was tested at two-month intervals (Table 8). In the experiment started on 17 September 1974, condition 5/5 was not included. For each treatment, 4 × 50 seeds (put on moist filter-paper in petri-dishes) were used. Germinated seeds were counted at intervals of 2–4 days during the first month and after that every 4–5 days.

Results

The results are shown in Table 9, expressed as percentages germinated seeds within 50 days. This period was chosen, because after that there was

Table 8. Temperature regimes used in the present germination experiments (17–9–1974 to 21–3–1975)

Temperature during dark period (16 hr)	Temperature during light period (8 hr)*	Referred to as:
5°C	5°C	5/5
5°C	10°C	5/10
5°C	15°C	5/15
5°C	20°C	5/20
5°C	25°C	5/25
5°C	30°C	5/30

* light provided by 4 fluorescent lamps (8 Watt, Philips, colour 33) per germination box

Table 9. Effect of storage conditions on the germination of *Cotula coronopifolia* and *Ranunculus sceleratus* seeds under various temperature regimes (expressed as percentage of seeds germinated within 50 days).

starting date of germination test	duration of storage (months)	treatment A (soil storage)						treatment B (cold storage)					
		5/5	5/10	5/15	5/20	5/25	5/30	5/5	5/10	5/15	5/20	5/25	5/30
Cotula coronopifolia													
17- 9-1974	fresh seed	.	69	98	98	100	99	.	69	98	98	100	99
22-11-1974	2	99	98	98	98	97	100	98	96	98	99	98	98
22- 1-1975	4	93	96	85	96	94	95	99	87	96	99	97	98
22- 3-1975	6	79	86	86	85	88	84	97	100	100	100	99	100
Ranunculus sceleratus													
17- 9-1974	fresh seed	.	28	90	90	89	92	.	28	90	90	89	92
22-11-1974	2	33	77	85	90	91	85	9	47	85	85	92	88
22- 1-1975	4	62	87	85	85	84	89	21	71	88	94	88	90
22- 3-1975	6	66	81	78	92	83	81	38	58	89	89	88	94

little or no germination. However, in a few cases (especially at lower temperatures) germination showed a gradual, but very slow, increase after the 50th day. Freshly harvested seeds of *C. coronopifolia* showed incomplete germination only under condition 5/10. Two months of storage resulted in complete germination for both treatment A and treatment B. After 4 months of storage, complete germination was maintained in treatment B, but in treatment A an increasing number of seeds failed to germinate. After 6 months this number reached about 15%. Under condition 5/15 about 90% of the freshly harvested seeds of *R. sceleratus* germinated. This percentage persisted in treatment B, but for A it became about 85% after 4 months of storage.

Germination of fresh seeds was low under conditions 5/10, and gradually increased at low temperatures (5/5 and 5/10). This increase was more pronounced for treatment A than for B. After storage for 6 months germination was still somewhat depressed, in treatment A only under condition 5/5, and in B under conditions 5/5 and 5/10.

Discussion

Germination of *R. sceleratus* seeds at low temperatures is low in the autumn but increases gradually during the winter, especially under soil storage at low temperatures. With this treatment they germinate mainly in the spring. *C. coronopifolia* seeds germinate mainly in the autumn and winter, because at low temperatures the germination of fresh seeds is only slightly depressed and after a two-month storage period is complete. Field observations indicate that the seedlings are not resistant to even light frost, which probably causes a high mortality. *R. sceleratus* seedlings – although they do not germinate frequently during the autumn and winter – seem to be frost resistant.

According to Thompson (1968), species from the Mediterranean region germinate at lower temperatures (under 10°C) than species from more northern latitudes (mainly above 10°C), as a result of which the former germinate mainly in winter and the latter in summer.

R. sceleratus and *C. coronopifolia* also have different seed dormancy. *R. sceleratus* seeds show a dormancy of about 10% directly after harvest, and this increases to about 15% after 4 month's storage in the soil. Fresh *C. coronopifolia* seeds do not show dormancy, but 6 months of storage in the soil induces a dormancy of about 15%. In this study non-germinating seeds were considered to be dormant, although their viability was not tested. (A positive indication for dormancy is provided by the fact that these seeds remained intact and did not rot.)

It is clear from all this that during the main germination period *R. sceleratus* seeds are partially dormant (spring), whereas *C. coronopifolia* seeds are not (winter). This means an advantage for the former species, because seed dormancy can be regarded as a spreading of risk. The latter species can avoid the disadvantages of germination at low temperatures,

lack of frost resistance, and absence of dormancy, if the seeds are submerged during winter and are subsequently exposed to dry conditions in the spring or summer. This is the situation prevailing where *C. coronopifolia* occurs in Zuid-Flevoland, viz. on the borders of shallow lakes which dry up during summer. A comparable situation is described for Langeoog (W. Germany), where Runge (1968) found the species around shallow pools. (The fresh-water situation in Zuid-Flevoland differs from the brackish conditions found at Langeoog and other growing sites along the German and Danish coasts.)

The moment of germination is critical for adequate seed production. An experiment on the influence of the sowing date on seed production showed that when the species is sown later than the end of June, no ripe seed is produced.

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5.3. GEOGRAPHICAL GROUPING OF SOME BIOLOGICAL PROPERTIES OF CARABID BEETLES IN THE NETHERLANDS (J. Haeck and R. Hengeveld)

When the reclamation of the youngest IJsselmeer polder, Zuidelijk Flevoland was started, Carabid beetles were chosen as object for the study of the dispersal and establishment of organisms in relation to environmental conditions. The Carabids were considered suitable for this purpose because one of their properties, the presence or absence of wings, might be of significance in this respect and could be investigated quite easily in large numbers of specimens. Moreover, this property of invading species can be compared with corresponding data from species on the mainland collected by the Biological Station at Wijster and Leiden University. It was to be expected that, especially during the first years after the reclamation of the polder, winged individuals would predominate. In the second place, we expected that the proportion of winged individuals would decrease over the years. Especially the rate of change of this proportion is of interest, from the point of view of both the spatial dynamics of populations and of population genetics. Indeed, during the first few year macropterous beetles were caught almost exclusively, but in the wing-dimorphic species the rate of change of the morphs differed from species to species and from site to site and was either too fast or too slow to be investigated successfully.

With respect to the question of the significance of wingedness for the dispersal power, we came to realize that no single property will be of significance, but that several properties in a complex may be operative. We therefore searched the literature for data on a number of properties that

would give the immigrants into the new polder their proper place as a distinct group of species within the total Carabid fauna of The Netherlands. Here, we will discuss only three of these properties: wing dimorphism, body length, and seasonal occurrence of the larvae, and discuss their inter-relationships, the geographical distribution patterns of two of these traits, and the possible ecological significance. The results of the examples given below do not represent a final evaluation, but may serve as indications for future research.

In the 1973 Progress Report we gave the frequency distribution of body length for the total species list of Carabids for The Netherlands in relation to those of the species caught in the IJsselmeer polders (Haeck & Hengeveld 1974). In the polder area a preponderance of the smaller species was found, which can possibly be explained either by a correlation between dispersal capabilities and size or between the habitats found in the polders and size, or by a combination of these two possibilities.

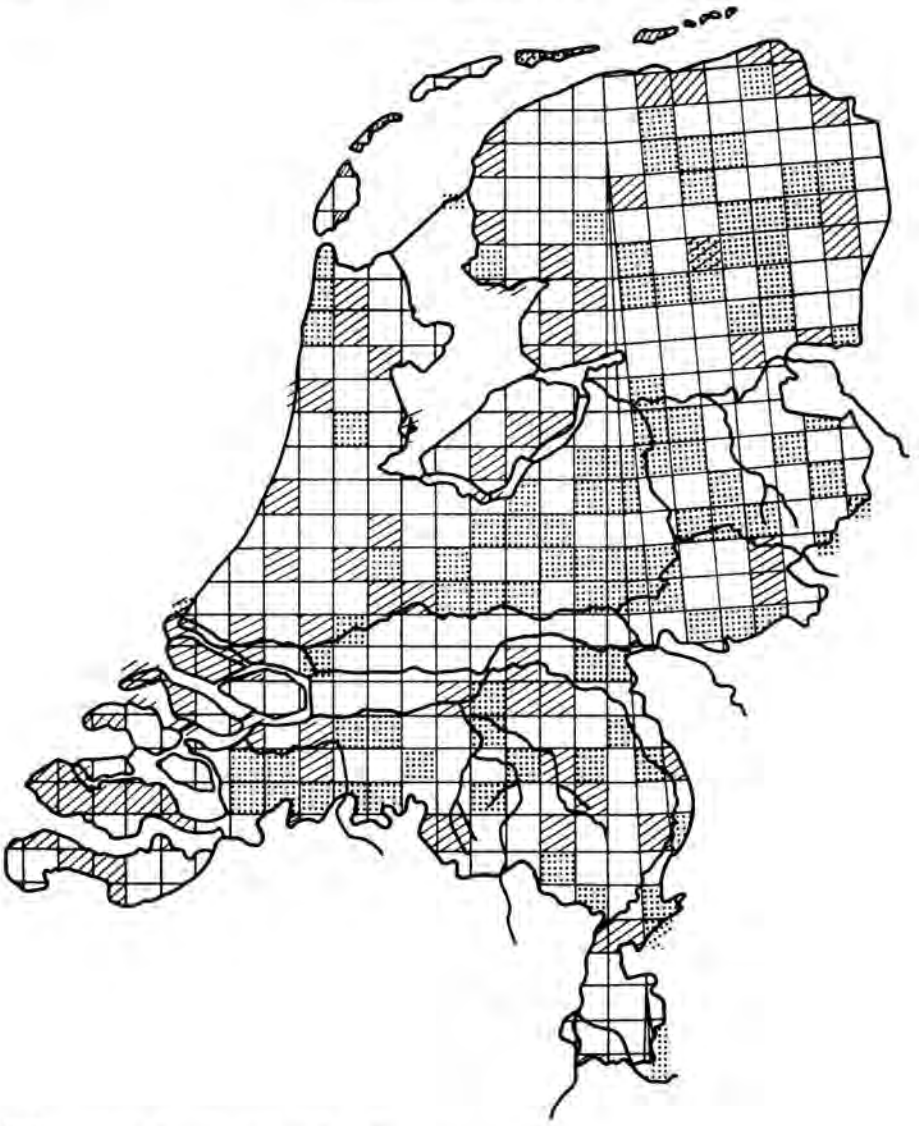
Calculation of the association between the body length and wingedness over the 373 species of the Dutch Carabid fauna, independent of the spatial distribution, shows a significant correlation:
$G = 58.48$, $df = 26$, $p < 0.0003$ (cf. Sokal & Rohlf 1969).

The association between these two variables remains significant when supplemented with a third one, the seasonal occurrence of the larvae (the larvae occurring either in winter or summer), whereas for the two new associations we find for size and larval occurrence $G = 107.06$, $df = 26$, $p < 5^{-12}$, and for wingedness and larval occurrence $G = 8.28$, $df = 9$, $p = 0.08$. Furthermore, there is a highly significant interaction term ($p < 10^{-14}$).

This year, an Atlas of Dutch Carabids giving distribution maps of all Carabid species based on material from private and museum collections, was completed. This survey enabled us to construct distribution maps of the above-mentioned properties. Fig. 5 shows the spatial distribution of body length and brachypterism. The body length category is arbitrarily chosen: it concerns the arithmetical means of the body sizes of all the species recovered in a 10×10 km^2 grid up to a certain value, 6 mm (i.e., roughly the median). Leaving out all squares with means greater than this value, we obtain the spatial distribution of this arbitrary category of species. The map shows that this category occurs mainly in the western part of the country.

The same map also shows the squares with a percentage of brachypterous species greater than 10 (again an arbitrarily chosen value, but roughly the mean for The Netherlands). This category occurs mainly in the eastern parts of the country, where the category of the small species is almost completely absent.

This spatial exclusion is in accordance with the above-mentioned association between wingedness and body length. A clue for an ecological interpretation of these phenomena may be found in some differences in condi-



mean length per grid < 7 mm.

percentage brachypterous species per grid > 10%.

Fig. 5. The spatial distribution of body length (hatched squares) and brachyptery (dotted squares) of carabid beetles in The Netherlands.

tions between the western and the eastern parts of the country. We feel that the greatest difference occurs in the soil conditions: the western region containing more moist clay and peat soils, the eastern more dry sands. In this connection it may be significant that the coastal sanddunes in the west contain some squares with percentages of brachypterous species greater than 10.

The smaller species are also the more mobile ones, and their larvae occur relatively more frequently in summer. This may be explained by the wetter environment, where the larvae cannot survive during the wet winter period (cf. Haeck & Reimerink 1972). Thus the combination of wingedness, smallness, and hibernation in the adult state may be characteristic for colonists of wet environments like the IJsselmeer polders.

These preliminary results indicate that it is worthwhile to characterize a species by several properties and that wide-scale distribution data contain ecological information relevant to the study of colonization.

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5.4. MICROMETEOROLOGY (Ph. Stoutjesdijk)

Microclimatic effects in the winter

Microclimatic effects which give rise to thermally privileged conditions are of special interest in the cold season. On a level surface the radiation intensities of the sun are low at this time of year and temperature effects are small too, but on a plane perpendicular to the direct solar radiation, intensities can be quite high. In the literature, average intensities of the direct solar radiation in clear country air at sea-level are given as 0.79 cal/cm² min with a solar elevation of 15° (December) and 1.22 cal/cm² min with an elevation of 30° (February). In good accordance with these values we found for the total (i.e., direct and diffuse) radiation measured perpendicular to the solar beam, values of up to 1.00 cal/cm² min for December and up to 1.30 cal/cm² min for February.

Fig. 6 shows surface temperatures as well as the corresponding air temperatures and the differences between surface and air temperatures (Δt). Even in December, rather high Δt values (up to 28°C) are reached; in February, Δt values of up to 40°C were observed.

The measurements made from December through February were done with a radiation thermometer on the bark of trees (mainly *Pinus silvestris*). In December, these bark temperatures were the highest found on natural surfaces. In February, a vertical surface is no longer ideal for reaching extreme temperatures: on a very steep edge of raw humus, temperatures lying 6–8°C higher than the corresponding bark temperatures (see Fig. 6) were occasionally observed. Because these steep raw humus surfaces rarely occur under natural conditions, for practical purposes we may still say that at this time of year the highest temperatures are measured on the bark of trees.

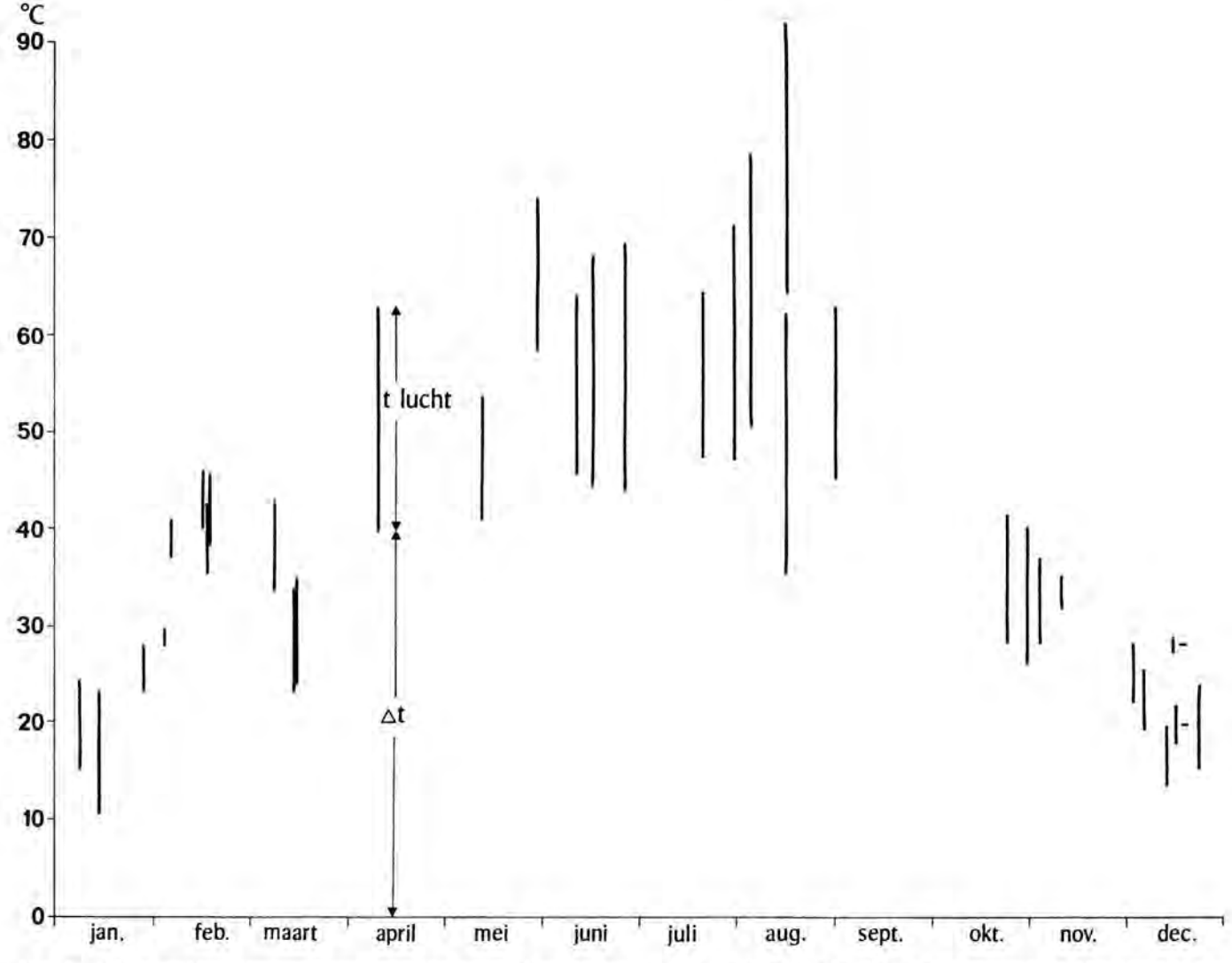

Fig. 6. High surface temperatures over the year. The top of the bar indicates the observed temperature, the length, the air temperature and the lower end the difference between surface and air temperature (Δt). When air temperatures are negative (indicated by a minus sign) Δt is indicated by the top and the surface temperature by the bottom of the bar.

At first sight the recorded temperatures may seem to be quite high, but in view of the radiation intensities involved they are rather low. The high bark temperatures were all observed on trees on the south-facing edge of pinewoods or in similar situations open to the sun and sheltered from the wind. In the winter, temperature effects on surfaces which are level or weakly inclined to the south are as a role quite small, but even in December the surface temperatures of litter may rise several degrees above air temperatures under favourable conditions. In February on weak slopes with litter, surface temperatures only slightly below those of pine bark are reached, and level surfaces show considerable effects, as can be seen in Fig. 7. It is relevant that both these observations and the December measurements referred to above were made at the southern edge of pinewoods. The existence of a thermally favoured situation at the southern edge of a wood or a dense hedge has been recognized by several authors, but was not adequately supported by suitable observations.

In this "warm fringe", as we shall call this counterpart of the "open shade" previously described, radiation conditions are more favourable than in the open field. In the open under a clear sky a level surface receives heat radiation from a hemisphere with an effective radiation temperature of 20–30°C below air temperature. When one half of this hemisphere is replaced by a canopy at or even slightly above air temperature, a relatively large amount of extra heat radiation is received. Shortwave radiation is only slightly affected, because the diffuse skylight is replaced by solar radiation diffused or reflected by the wood. We measured in the fringe a total radiation gain of 0.06–0.08 cal/cm^{2}min and sometimes more. Energy balance considerations show that this would account for a temperature difference of 3–5°C between identical surfaces in the fringe and in the open.

Identical surfaces are hard to find, but where a comparison could be made the difference was higher, i.e., 10°C. Since the radiation gain is present day and night with clear skies and to a small degree also with a cloud cover, we assume that there can be a cumulative effect: because the surface at the edge cools less by night and collects less dew, it is in a better position to warm up in the sun than a freely exposed surface. Furthermore, the reduced air movement in the fringe is of importance and probably the reduction of rainfall as well.

The question arises whether the observed temperature effects, which are considerably but unpredictable and infrequent, have any biological meaning. First, there is the visual evidence. Flies bask in the sun on heated surfaces until late in December. In February flies, ladybirds, spiders, ants, lizards, and vipers can be seen basking in the sun. Apparently they select the most favourable situation. Small insects and spiders, which form as it were part of the surface, can undoubtedly attain temperatures only a few degrees below that of their substrate, as indicated by measurements with similarly sized probes. Lizards and vipers, due to their size, are not so directly coupled to the temperature of the substrate, but by flattening their

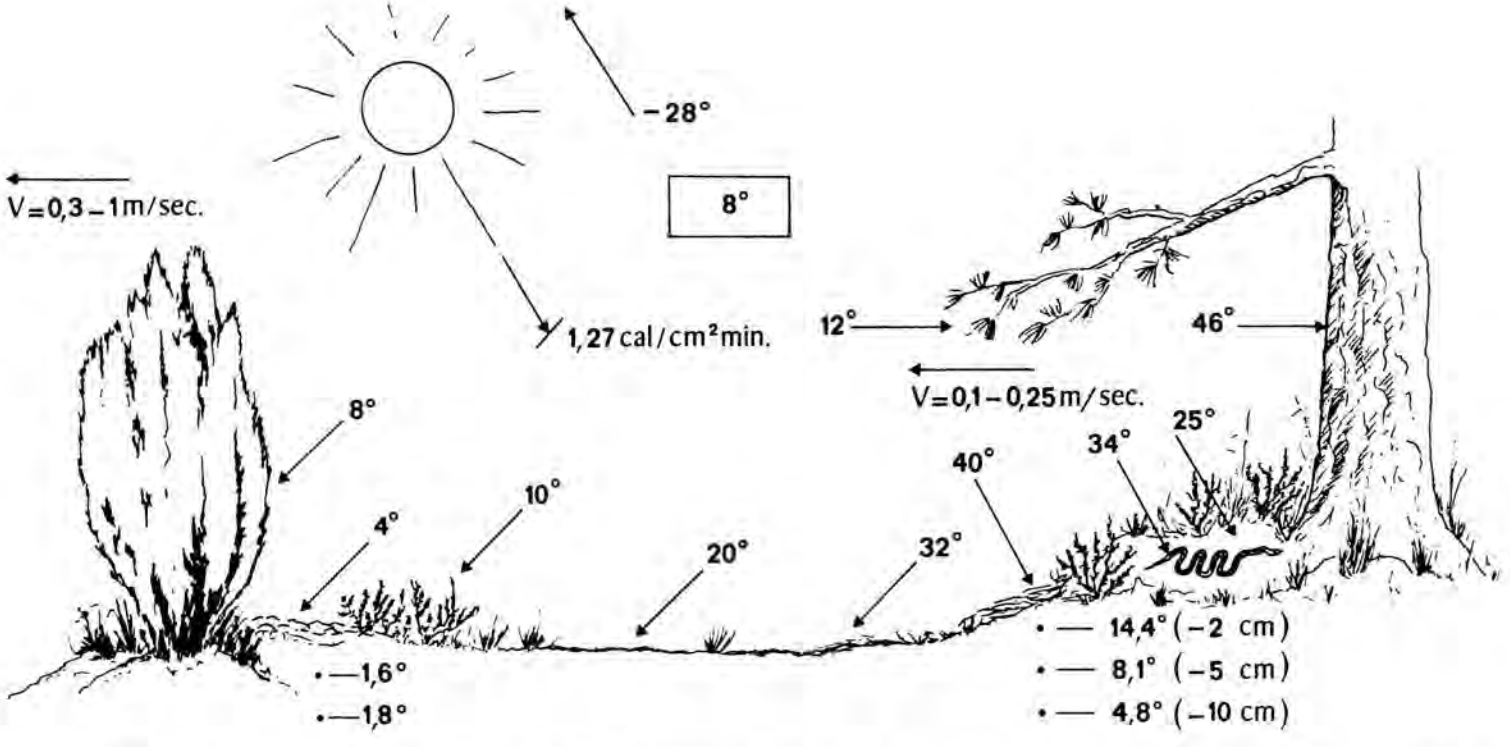

Fig. 7. Schematic cross-section of the southern edge of a pinewood, showing the micrometeorological situation on the middle of a sunny day at the end of February. Wind velocity (measured by a hot-wire anemometer) is indicated by V. Effective radiation temperature of the sky: −28 °C. Solar radiation measured perpendicular to the solar beam: 1.27 cal/cm² min. Air temperature: 8°C.

body and orienting it perpendicular to the direct solar rays they can attain temperatures lying as much as 25°C above the air temperature, at least at the surface of the body.

That basking at the end of the winter is essential for ants *(Formica polyctena)* and initiates important physiological changes was shown by Kneitz (1964), and Viitanen (1967) considers basking essential for spermatogenesis in vipers. In the case of poikilothermous animals it seems plausible that short periods of an elevated temperature permitting high locomotory activity, can be of great importance. Consequently, limited weight is to be attached to average values. Mean temperatures may have some meaning where processes of growth and development are concerned; here, a linear relationship between rate and temperature may be a good approximation (Precht *et al.* 1973; Went 1957).

It is selfevident that small animals which can select the most favourable position can exploit the possibilities of the microclimate much better than sessile organisms such as plants. It is useful to estimate the mean temperature excess a small animal can attain in this way. The assumption that when the sun shines a mean temperature excess of 20°C above ambient air can be maintained, leads to an average temperature excess of 2°C for February with 70 sunshine hours. For plants the estimate must be much lower, due to their fixed position and even more so to their growth form, higher reflectivity, and transpiration. Living plants rarely attain a temperature lying 10°C above that of the ambient air in winter. An upper limit of 0.5°C of the mean temperature excess due to solar heating seems a reasonable estimate.

Solar heating is not the only source of temperature differences between plants or animals. Long-wave radiation conditions give rise to temperature differences which are smaller than those due to solar radiation. However, because these differences are present night and day in cloudless weather, they may have a stronger effect on the average temperature than solar radiation, at least for plants. For animals, the ability to retreat underground at night complicates the situation further.

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6. Ecological dune research (Biological Station "Weevers' Duin")

6.1. GERMINATION AND CULTURE EXPERIMENTS WITH CALCAREOUS AND ACIDIC DUNE-SAND SUBSTRATES ON THE CALCICOLOUS *Cynoglossum officinale* L. (A. H. J. Freijsen)

Introduction

The plant species *Cynoglossum officinale* L. *(Boraginaceae)* was studied in a series of germination and culture experiments started in 1974 (Freijsen 1975). The experiments were carried out to determine the significance of two environmental factors, lime and nitrogen. In The Netherlands, *C. officinale* is restricted almost entirely to the coastal areas with calcareous dunes. According to the literature, the species occurs in other countries on calcareous soils as well (e.g. Clapham *et al.* 1962; De Langhe *et al.* 1973; Fournier 1961). Within The Netherlands distribution area, the species behaves as a nitrophilous plant.

Natural dune sands from The Netherlands were used as substrate. Three types of soil were applied: 1. calcareous dune sand from Voorne ($CaCO_3$ 6.0%; organic matter 1.0%); 2. dune sand from the acidic dunes north of Bergen (pH 4.9; organic matter 0.4%); 3. slightly calcareous sand from the transitional zone between the calcareous and the acidic dunes near Bergen ($CaCO_3$ 0.5%; organic matter 0.6%). For further details, the reader is referred to Freijsen (1975).

The main results of the experiments, with dune-sand substrates are briefly reported here. In addition to these experiments, a water culture experiment was carried out to assess the response of *C. officinale* to various concentrations of Ca.

Germination experiments

In all experiments the germination percentage was lowest on acidic dune sand, i.e., between 5 and 30 per cent, and the germination time the longest There was also a difference between the calcareous and the slightly calcareous sands. Within each experiment, more seeds of *C. officinale* germinated on the calcareous sand from Voorne. In most of the experiments seeds were incubated in sand with a water content of 10 per cent by weight. At a water content of 5 per cent the germination response was essentially the same.

Two experiments were specifically set up to investigate the poor germination of *C. officinale* on the acidic dune sand. The original properties of the sand were changed by the application of $CaCO_3$, Na_2CO_3, or $CaSO_4$. In the first two cases the pH was raised to about 8. In none of these treatments did the low germination percentage increase. It is notable that juvenile plants showed healthy growth on the limed sand but not on acidic sand with $CaSO_4$ or Na_2CO_3.

In the literature I could only find one example of a direct negative influence of an acidic soil reaction on germination. Rorison (1967) described

a decrease of the germination percentages of the calcicoles *Scabiosa columbaria* and *Erigeron acer* on acidic substrates. In general, seed germination seems to be independent of the soil reaction (e.g. Knapp 1967). It is obvious, however, that in general the establishment of the seedling is strongly affected by chemical soil conditions. The low germination percentages of *C. officinale* at low pH values may be called exceptional.

Because few data from comparable germination experiments were found in the literature, some additional experiments with other plant species were carried out. The experimental procedure was the same as for *C. officinale*. The germination of the calcicolous *Hypericum perforatum* L. varied, with the highest percentage on the slightly calcareous dune sand and the lowest on the acidic sand. Seeds of the calcicolous *Centaurium minus* L. and *Melandrium rubrum* (Weig.) Garcke germinated equally well on the three types of dune sand. In these two species the development of seedlings on acidic sand was disturbed.

Sand culture experiments

In three culture experiments rosettes of *C. officinale* were grown on the three types of dune sand to find out whether the plants would show differences in dry matter production and chemical composition, and, if so, whether such differences were correlated with the $CaCO_3$ and N levels in the substrate.

The following results of these pot experiments are worth mentioning. On the acidic dune sand, seedlings did not grow at all. Moreover, these seedlings had an unhealthy appearance. The rosettes cultured on the calcareous and the slightly calcareous substrates developed symptoms of nitrogen deficiency in the course of the experiments. Their old leaves became yellow. The N content of the leaves of the harvested plants of these treatments was extremely low. The growth of the *C. officinale* plants was clearly determined by the N supply from the dune sand. The plants on the calcareous sand from Voorne attained a higher dry weight as a consequence of the higher organic matter and nitrogen contents of this sand. The Ca contents of the plants grown on both the calcareous and the slightly calcareous dune-sand substrates were higher than those found in plants growing naturally in the dunes of Voorne, which means that the growth reduction on the slightly calcareous sand was not caused by Ca deficiency. The differences in productivity between the calcareous and the slightly calcareous sand were not found when a complete nutrient solution was added to the substrate.

Conclusions

The low percentage of germination of *C. officinale* on the acidic dune sand is related in some way to the acidic soil reaction of this sand. Because liming did not improve the germination, it may be concluded that a high concentration of H-ions is not the only important factor. On the calcareous

dune sand from Voorne the germination percentages were always higher than on the slightly calcareous sand from Bergen. Preliminary results of more recent experiments show that the germination of *C. officinale* is stimulated by nitrates. The differences in germination behaviour on the calcareous and slightly calcarous dune sands are probably related to the different levels of organic matter and nitrogen in these soils.

The low germination percentage and the completely disturbed growth of *C. officinale* on the acidic dune sand substrate explain why this species is absent on the acidic coastal dunes in The Netherlands and on other acidic sandy soils. The observed differences in germination and in vegetative growth between the calcareous and slightly calcareous substrates, which also differed in organic matter content, help to explain the preference of *C. officinale* for nitrogen-rich environments in the more or less calcareous coastal dunes of The Netherlands.

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6.2. EFFECTS OF COMPACTION OF DUNE SAND ON THE EMERGENCE AND DEVELOPMENT OF FOUR *Plantago* SPECIES IN EXPERIMENTAL PLOTS (C. W. P. M. Blom)

Introduction

The research on the influence of soil compaction on the occurrence of some *Plantago* species on coastal sand dunes was continued with sowing experiments in open plots. In the dune area, the mechanical compaction of the soil is caused mainly by trampling by people and animals; the effects of trampling and soil compaction on plant growth are combined (see also: Liddle & Greig-Smith 1975 a, b). The aim of studies done in experimental plots is to investigate these effects separately.

The earlier studies (Blom 1972, 1973, and 1974) on the influence of trampling and soil compaction on seedling emergence concerned growth-cabinet experiments with several soil-moisture levels and sowing experiments on dune soils covered by vegetation. In the present experiments the *Plantago* seeds were sown in open bare plots with loose, moderately com-

pacted, or compacted dune-sand soils in which the factor trampling is absent. Thus, the influence of soil compaction occurring under natural climatological circumstances on the emergence, establishment, and development of four *Plantago* species could be studied. The relationship between the results of these open plot experiments and those of the preceding experiments will be discussed.

Materials and methods

The experiments were carried out in an experimental garden in the dune area of Voorne. The original humic soil was removed to form a trench with a depth of 1 metre, a width of 3 metres, and a length of 18 metres, which was then divided into six compartments, each measuring 9 square metres. The compartments were separated by concrete walls and filled with the same dune sand as had been used in the preceding growth-cabinet experiments (organic matter content 0.5%, pH 9.0). The sides of the trench were covered with plastic foil to prevent contact between the original humic soil and the dune sand.

Compaction was obtained by ramming down the substrate in layers of 20 cm each; after packing of each layer the surface was scraped to give a homogeneous soil profile. Two compartments were filled with loose dune sand (penetrometer value <0.1 kg/cm^2; pore volume 45%); in a second pair the soil was brought to maximal compaction (penetrometer value 10 kg/cm^2 at a depth of 2 cm; pore volume 39%); in the remaining two compartments the resistance of the soil 2 cm beneath surface was 5 kg/cm^2 and the pore volume in the upper layers 42%. The compaction of these plots was therefore comparable with the field situation and earlier experiments carried out in the growth cabinets.

The ground-water level in the plots was about 70 cm below the soil surface and the soil moisture in the top layers depended on natural rainfall. On both sides of the midline of each plot, 100 seeds each of *P. lanceolata*, *P. coronopus*, *P. major*, and *P. media* were sown separately on 11 August 1972, giving four replicates per species. The seeds were covered with 3 mm of dune sand which was given the same density as the underlying layer. During the experiment, the emergence and establishment of seedlings and the growth and the flowering capacity were investigated. The penetrometer values were determined just before sowing and at the end of the experiment.

Results

The results of the emergence of *Plantago* species sown in the experimental field plots are given in Fig. 8a–d. Graphs a and b show the percentages of emerged seedlings of *P. lanceolata* and *P. coronopus* in 1972 and 1973. Since no seedlings of *P. major* and *P. media* emerged in 1972, graphs c and d show the percentages of emergence in 1973 and 1974.

Four days after sowing, 20 per cent of the seeds of *P. lanceolata* and about 15 per cent of those of *P. coronopus* had emerged on the plots with

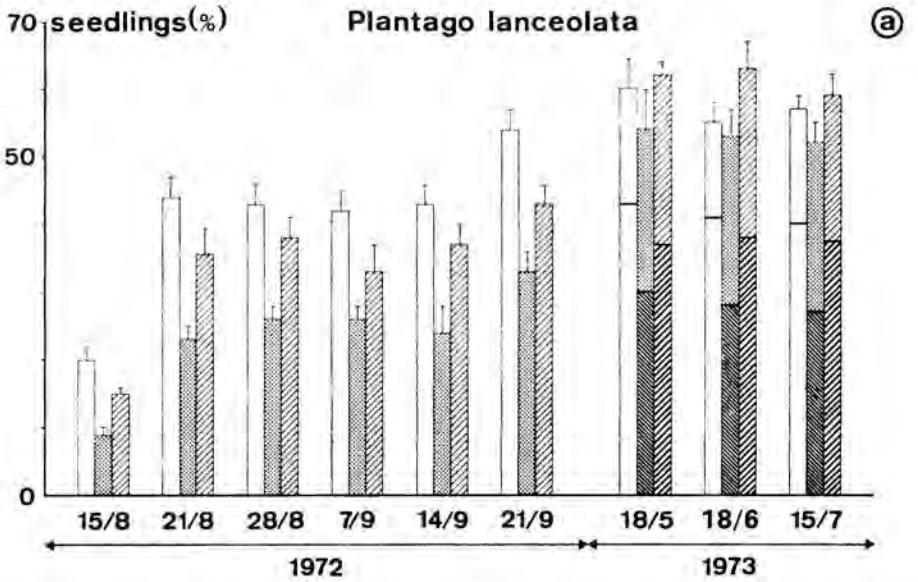

Fig. 8a.

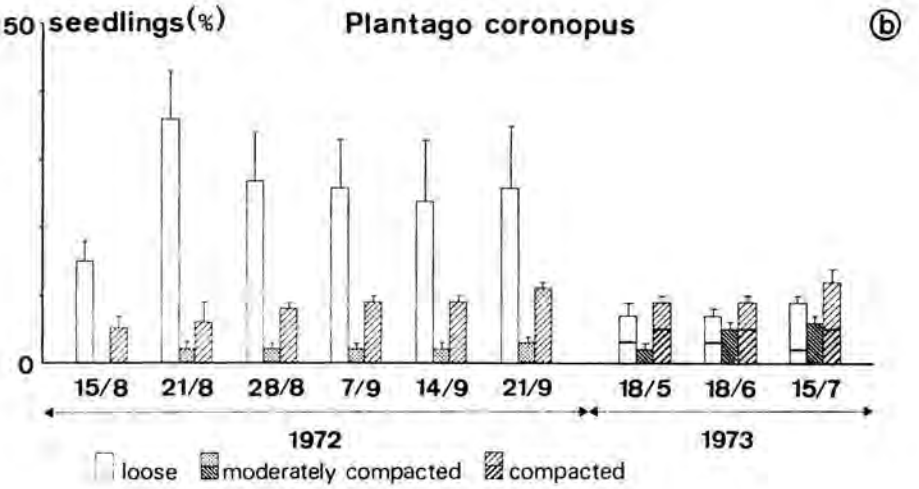

Fig. 8b.

Fig. 8 (a–d). The influence of soil compaction on seedling emergence from buried *Plantago* seeds in experimental plots in the field (mean values of four comparable plots). For the columns on the right a distinction was made between newly emerged plants and plants of the preceding year, the upper part of the column representing the former and the lower part the latter. The vertical bars represent the standard deviation.

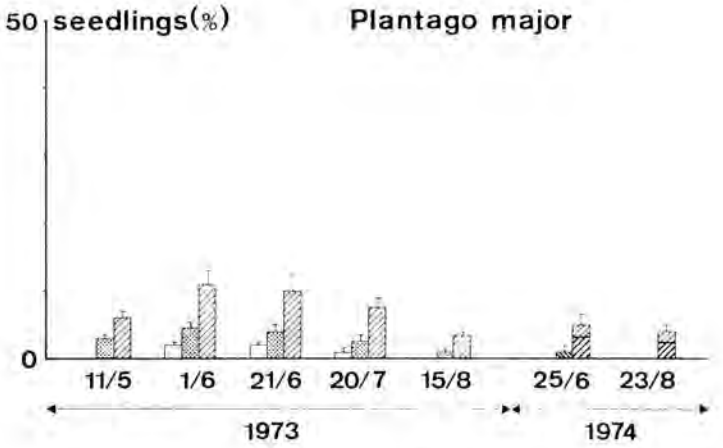
50
seedlings(%)
Plantago major
©
0
11/5
1/6
21/6
20/7
15/8
25/6
23/8
1973
1974

Fig. 8c.

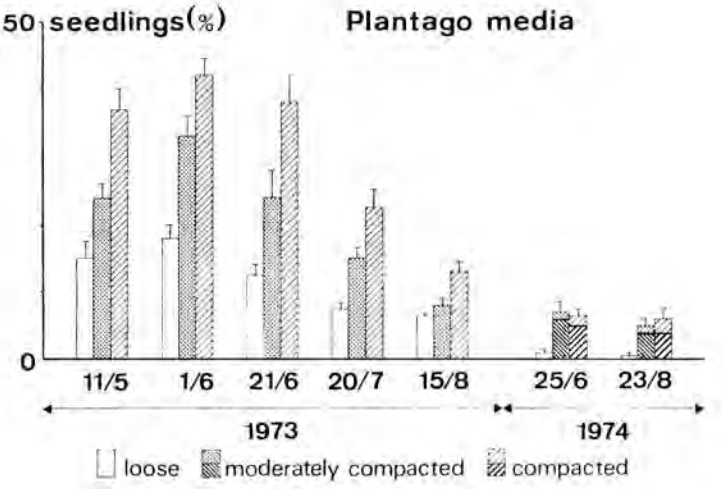
50
seedlings(%)
Plantago media
ⓓ
0
11/5
1/6
21/6
20/7
15/8
25/6
23/8
1973
1974
loose
moderately compacted
compacted

Fig. 8d.

the loose soil. On the moderately compacted soil, only emergence of *P. lanceolata* (9%) was observed at this time. On the compacted soil, on average 15 per cent of *P. lanceolata* and 5 per cent of *P. coronopus* had emerged. Six days later, the emergence of *P. lanceolata* and *P. coronopus* on all plots reached a level which remained constant until the second part of October 1972, although some *P. coronopus* seedlings on the loose soils died off at the end of August. In the second part of October more seedlings of these species emerged. In 1972, the highest percentages of *P. lanceolata* and *P. coronopus* seedlings occurred on the loose soil and the lowest percentages on the moderately compacted substrate. The 1973 results are also shown in Fig. 8 (a and b), where a distinction is made between plants present since 1972 and seedlings originating from seeds sown in 1972 and emerged in 1973. For *P. lanceolata* and *P. coronopus*, the differences in total numbers of living plants between the various plots were smaller in 1973 than in 1972.

In 1973 and 1974, significantly more seedlings of *P. major* and *P. media* occurred on the compacted than on the loose substrates (Fig. 8, c and d). Initially, *P. major* occurred in very small numbers on the loose soil; at the end of 1973, this species disappeared completely from this substrate and at the end of 1974, *P. major* was only present on the compacted soil. On all plots the total numbers of *P. media* seedlings were higher than those of *P. major*.

Besides the counting and micro-mapping of the individuals on the plots, some other features were noted. Table 10 gives for the various soils the mean values of rosette diameters of the non-seedling plants, the mean percentages of flowering plants, the mean numbers of spikes, and the mean length of the peduncles and spikes. In this table the values for August 1973 and 1974 are given, because the maximal size and flowering capacity was achieved in these months. In 1975, the nutrients in the plots became depleted, which resulted in small and yellow plants. During this experiment *P. lanceolata* and *P. coronopus* flowered; *P. major* and *P. media* occurred only in the vegetative form. As can be seen from Table 10, the largest rosettes occurred on the loose substrates; between the moderately compacted and compacted soils there were only small differences in rosette diameter. In general, the most flowering plants were found on the loose soils, but in 1974 higher numbers of *P. coronopus* flowered on the moderately compacted substrates. For both *P. lanceolata* and *P. coronopus* the highest numbers of spikes per individual and the largest peduncles and spikes were found on the loose soils. For *P. lanceolata* no important differences in numbers and lengths of spikes were found between the moderately compacted and compacted soils; in 1974, the numbers of spikes of *P. coronopus* were distinctively higher on the compacted than on the moderately compacted substrates.

The soil moisture varied widely in the experimental plots (mean value about 5 per cent by volume in the upper layers), and germination generally occurred after a spell of rainy days.

Table 10. Some features of *Plantago* species in relation to soil compaction*

	August 1973			August 1974		
	L	M	C	L	M	C
A. Mean rosette diameters of the non-seedlings (cm)						
P. lanceolata	25.0 ± 4.1	13.2 ± 2.2	12.7 ± 2.2	15.0 ± 1.6	8.0 ± 1.3	6.7 ± 1.3
P. coronopus	17.7 ± 3.1	6.7 ± 1.6	7.8 ± 3.5	13.2 ± 4.1	7.2 ± 1.5	6.4 ± 1.2
P. major	–	2.7 ± 1.1	1.8 ± 0.6	–	–	2.1 ± 0.9
P. media	1.6 ± 0.9	1.3 ± 0.8	1.2 ± 0.8	3.1 ± 1.1	2.7 ± 1.0	1.8 ± 0.7
B. Flowering of the total numbers of plants per species (%)						
P. lanceolata	22.0	3.9	0.3	87.5	58.7	47.5
P. coronopus	67.5	31.2	34.3	85.1	93.9	82.6
C. Mean numbers of spikes per individual						
P. lanceolata	7.2 ± 2.5	3.1 ± 2.7	1.3 ± 1.0	23.5 ± 3.0	6.5 ± 1.2	5.5 ± 1.7
P. coronopus	19.3 ± 3.4	4.5 ± 2.5	4.2 ± 3.1	28.5 ± 1.9	9.5 ± 2.1	20.3 ± 3.6
D. Mean length of the peduncle + spike (cm)						
P. lanceolata	30.5 ± 3.1	21.0 ± 3.4	18.1 ± 3.9	30.3 ± 1.2	15.3 ± 2.9	13.2 ± 2.4
P. coronopus	14.7 ± 2.1	6.8 ± 1.9	5.0 ± 2.2	13.3 ± 2.5	6.7 ± 1.2	7.3 ± 1.7

* Mean values and standard deviation. L = loose; M = moderately compacted; C = compacted

Penetrometer measurements were made twice: just before sowing and in September 1974. The measurements (Fig. 9, a and b) were made at depths of two, five, and ten centimetres at similar soil-moisture levels in 1972 and 1974. At a depth of two centimetres the differences between the various plots were nullified in 1974, whereas in all series the mechanical resistance at five centimetres had increased. At a depth of ten centimetres the original degree of compaction still existed except in the plots with the loose substrates, in which a slight degree of compaction had occurred.

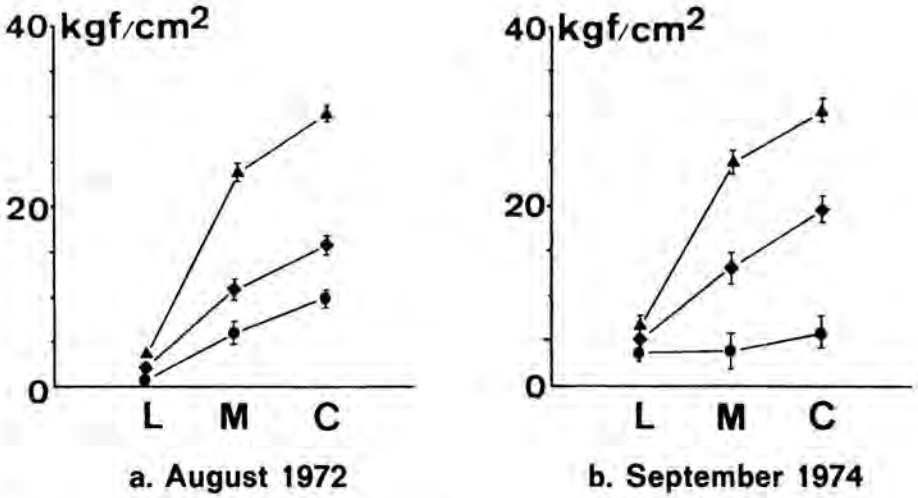

Fig. 9. Penetrometer values measured at three depths in the field plots just before sowing (August 1972) and at the end of the experiment (September 1974): ● 2 cm; ♦ 5 cm; ▲ 10 cm. The vertical bars represent twice the standard deviation. L = loose soil; M = moderately compacted soil; C = compacted soil.

Discussion

In experimental plots in the field *P. lanceolata* and *P. coronopus* emerged sooner and in higher percentages on bare loose dune-sand soils than on bare compacted or moderately compacted dune-sand substrates, whereas *P. major* and *P. media* occurred mainly on plots with compacted soils. About a year after sowing, (i.e., in 1973) the total numbers of living plants of *P. lanceolata* and of *P. coronopus* were approximately the same on all plots. This was probably due to the decreasing compaction in the upper layers of the moderately compacted and compacted soils. In 1974 and 1975, the most *P. lanceolata* seedlings emerged on the loose soils and the most *P. coronopus* seedlings on the moderately compacted and compacted soils; the highest numbers of seedlings emerged on the plots with the highest flowering rate. In 1974 and 1975, the compaction of the soil was of little importance for emergence, because at that time it was about the same in the upper layers (2 cm) (see Fig. 9). The differences in germinational

behaviour in the experimental plots between *P. lanceolata* and *P. coronopus* on the one hand and *P. major* and *P. media* on the other were most probably caused by the combined action of mechanical soil resistance and soil moisture. In these bare sand plots the soil moisture was, in general, very low. Because *P. lanceolata* and *P. coronopus* germinated better than *P. major* and *P. media* at these low levels of soil moisture, it may be supposed that for the latter two species, under these conditions, soil moisture is more limiting for germination than is the case for the former. This conclusion is supported by the observation that the emergence of *P. major* and *P. media* was higher on the compacted than on the loose soils, the water availability being greater on compacted soils.

The results show that the mechanical resistance was the most important unfavourable factor for the emergence of *P. lanceolata* and *P. coronopus*. The low levels of emerged seedlings on the moderately compacted soils are possibly due to the combination of two unfavourable factors, namely a certain degree of mechanical soil resistance and a relatively low water availability.

For the *Plantago* species under study, the rate of compaction proved to be of great importance to the growth and flowering; an increase in soil compaction causes a decrease in rosette diameter and length of peduncles and spikes, and, particularly for *P. lanceolata*, a decrease in the flowering rate. In 1974, the percentages of flowering *P. coronopus* plants were the highest on the moderately compacted soils and higher numbers of spikes per individual occurred on the compacted than on the moderately compacted substrates. The differences in growth of the species between the various plots can be ascribed on the one hand to the higher penetration capacity of plant roots in loose soils (Blom 1972, 1973; Scott Russell & Goss 1974) and on the other to the lower uptake by plants of certain nutrients in compacted soils (Murty 1964).

In conclusion it may be said on the basis of the results of preceding experiments and those described in this report that with respect to seedling emergence and establishment on soils with a high mechanical resistance *P. major* is best adapted, followed by *P. media*, *P. lanceolata* being the least well adapted. *P. coronopus* seems to be more or less indifferent to mechanical resistance.

The emergence of *P. major* and *P. media* is very vulnerable with respect to adverse moisture conditions. This may explain the absence of these species in loose but dry sandy soils; *P. coronopus* and *P. lanceolata* are better adapted to these soil conditions.

In the near future, studies on experimental plots under field conditions will be continued with manured dune-sand soils, and trampling experiments will be performed to investigate the combined effects of trampling and soil compaction on the growth of the same four *Plantago* species.

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6.3. THE RELATIONSHIP BETWEEN NODULATION AND N_2-FIXATION IN *Hippophaë rhamnoides* L. (P. A. I. Oremus)

Introduction

Field observations have shown that the nodulation of *Hippophaë rhamnoides*, in terms of number of nodules per plant, is highly variable (Akkermans 1971; Oremus 1971; 1975; Stewart & Pearson 1967). The present investigation concerned the effect of the degree of nodulation on the N_2-fixation activity of these nodules. To determine this activity, the acetylene-reduction assay was used.

Materials and methods

Groups of five pre-cultivated *H. rhamnoides* plants (Oremus 1975) were placed in 250 ml glass jars provided with 200 ml nitrogen-free Hoagland II solution (pH 6.8) and 10^{1}, 10^{0}, $10,^{-1}$ or 10^{-2} mg ground nodule material. The nodule suspension was prepared from one living nodule collected in the field. The jars were placed in a phytotron under the following climatic conditions: temperature 23°C, air humidity 70%, light intensity at culture level 30,000 lux, and 16 hours of illumination alternating with 8 hours of darkness. After 7 days each plant was placed in a 1-litre pot provided with 100 gram perlite and 350 ml Hoagland II (−N). The plants were grown in a glasshouse for 18 weeks and maintained on a 16-hour photoperiod at 27°C and an 8-hour dark period at 20°C. Light was supplied by sunlight. Water lost by evaporation was replenished daily. The plants were transferred to the phytotron 24 hours before the activity of the nodules was measured.

Table 11. Biomass of *Hippophaë rhamnoides* plants and acetylene-reducing activity of the nodules

number of nodules	nodule dry weight (g)	plant dry weight (g)	acetylene-reducing activity (μmoles C_2H_4/h)	acetylene-reducing activity per gram nodule dry weight (μmoles C_2H_4/g/hr)	N content of plant plus nodules (g)
7	.002	0.128	0.03	20.00	.003
8	.000	0.175	0.03	75.00	.004
9	.004	0.154	0.12	32.63	.002
3	.141	3.483	5.20	36.87	.117
3	.144	2.220	6.04	42.04	.063
2	.041	0.735	0.02	0.49	.012
6	.099	2.193	3.17	32.03	.058
3	.161	2.765	5.04	31.30	.088
11	.163	4.039	6.09	37.46	.006
22	.116	3.414	0.15	1.30	.077
18	.212	5.419	6.48	30.57	.162
10	.153	4.111	5.16	33.72	.119
19	.198	3.483	6.23	31.49	.251
23	.128	4.270	5.44	42.57	.120
18	.172	3.618	8.23	47.75	.115
23	.229	4.552	8.29	36.16	.288
8	.109	3.146	3.38	30.99	.200
18	.130	3.494	5.12	39.51	.107
23	.163	3.872	3.65	22.42	.102
21	.150	3.946	3.85	25.67	.123
16	.134	3.934	3.88	28.96	.118
9	.144	3.165	4.48	31.16	.095
28	.100	3.839	5.18	28.74	.153
68	.134	4.059	3.87	28.90	.139
98	.146	4.392	4.35	29.82	.150
73	.126	3.972	5.57	44.16	.209
17	.161	2.960	7.74	48.15	.187
34	.109	3.996	6.43	59.14	.108
23	.153	4.213	4.79	41.25	.131
61	.157	4.244	4.58	29.17	.140
25	.101	3.839	4.90	48.40	.111
60	.113	3.427	5.75	50.82	.104
66	.097	3.936	5.04	52.21	.074
63	.105	4.333	6.28	59.72	.091
65	.111	2.422	2.74	24.65	.084
31	.075	2.051	5.22	69.53	.071
50	.152	4.205	0.01	0.09	.134
20	.104	2.801	5.56	53.69	.091
80	.107	4.725	2.64	24.72	.250
57	.134	3.300	4.57	34.08	.102
34	.084	2.422	2.61	31.29	.131
64	.146	5.604	9.46	64.77	.167

Table 11. (Continued)

48	.122	3.364	3.13	25.66	.119
111	.097	3.979	6.26	64.56	.121
69	.184	3.988	7.15	38.92	.129
44	.125	2.365	6.14	49.29	.053
75	.088	3.414	4.80	54.50	.168
98	.126	4.735	7.02	55.61	.127
60	.112	4.097	2.24	20.01	.122
75	.099	3.413	6.28	63.27	.108
58	.094	3.568	4.77	50.89	.109
70	.119	3.980	5.06	42.55	.129

Each plant was incubated in a 7-litre Perspex vessel covered by a Perspex lid sealed with vasoline. Acetylene was injected into the vessel with a polythene syringe to the desired partial pressure of acetylene (0.1 atm). At a pC_2H_2 of 0.1 atm the acetylene reduction is not competively inhibited by N_2 (Akkermans 1971). Saturation of nitrogenase in intact nodules was already obtained at pC_2H_2 of 0.04 atm. The apparent K_m for nitrogenase was determined to be 0.010 atm. (Oremus, unpubl.). The acetylene was purified by flowing the gas through 4 successive filter tubes, viz. a) a soda-asbestos filter; b) a water filter; c) a H_2SO_4 (80% v/v) filter; and d) a water filter (Akkermans 1971). After incubation for 5 minutes, gas samples (200 μl) were drawn at given intervals over a 35-minute period. Ethylene production was measured with a Becker type 407 gas-chromatograph equipped with a flame-ionization detector. Nitrogen was used as carrier gas, at a flow rate of 15 ml/min. The column (120 cm long, 3.5 mm inner diameter) was packed with Porapack R and maintained at 60°C.

Results

The results are shown in Table 11. The mutual correlation of a number of factors was calculated. When significance was found, the regression coefficient was determined. These coefficients are shown in Table 12. There is no significant correlation between the number of nodules per plant and the total dry weight of these nodules (row 1). The same holds for the number of nodules and the acetylene reduction activity of these nodules (row 2). There is a significant positive correlation between the number of nodules and the activity per gram dry nodule weight (row 3). If the number of nodules per plant increases, the nodules remain smaller and the activity per unit of weight increases slightly. However, this increase is so small (row 3, column 3) that the total activity of the nodules does not change (row 2, column 1). The correlations between total nodule dry weight on the one hand and plant dry weight, total nodule activity per plant, and total N content of the plant plus nodules on the other, are all significant ($P \ll 0.001$). No significant correlation was found between total nodule dry weight and acetylene reduction activity per unit of nodule dry weight.

Table 12. Correlation and regression coefficients

factors		(1) correlation coefficient	(2) regression coefficient	(3) 95% confidence interval for βyx	
x	y	r	b_{yx}	lower limit	upper limit
(1) number of nodules	nodule dry weight (g)	0.008			
(2) number of nodules	activity (μmoles C_2H_4/h)	0.214			
(3) number of nodules	activity/g nodule dry weight (μmoles C_2H_4/g/h)	0.370"	0.153	−0.002	0.308
(4) number of nodules	N content (g)	0.282'			
(5) nodule dry weight	plant dry weight (g)	0.725"	18.546	13.559	23.533
(6) nodule dry weight	activity	0.621"	29.906	19.191	40.621
(7) nodule dry weight	activity/ g nodule dry weight	−0.167			
(8) nodule dry weight	N content	0.657"	0.820	0.555	1.085
(9) activity	N content	0.471"			

' $r_{0.05} = 0.273$
" $r_{0.01} = 0.354$

Discussion

The results of this experiment show that the acetylene reduction activity of young *H. rhamnoides* nodules is independent of the number of nodules present and that the activity is positively correlated with the total nodule dry weight. This dry weight is not correlated with the number of nodules present. Therefore, it is conceivable that differences in nodulation observed in the field (Stewart & Pearson 1967; Akkermans 1971; Oremus 1971,

1975) are not of great importance for the N_2-fixation and growth rate of the plant: the amount of N_2 fixed by a large number of relatively small nodules is not necessarily higher than that fixed by a small number of relatively large nodules. Consequently, the growth rate of the nodules is of great importance for the development of the plant, and it is useful to study the factors that influence this growth rate.

According to the present results, 120-day-old nodules reduce 19.2 to 40.6 μmol C_2H_2/g nodule dry weight/hr. Results of another experiment show that 60-day-old nodules reduce 73.1 to 151.8 μmol C_2H_2/g/hr. So it is clear that the acetylene reduction activity per unit nodule weight decreases rapidly when the nodules grow older; this is confirmed by the results of other experiments not mentioned here. The wide varation in the age distribution of field-grown nodules, the possible variation in the growth rate if nodules in different habitats, combined with the results of this experiment, make it clear that the activity of field-grown nodules is highly variable (Akkermans 1971). For these reasons it is extremely difficult to study the environmental factors affecting the development and reducing activity of field-grown nodules. This problem can be solved by measuring the reduction activity of the same nodules in the course of time and by comparing these activities under different environmental conditions. Such experiments are in progress in our laboratory.

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6.4. ECOLOGY OF THE ROOT-NODULE ENDOPHYTE OF *Alnus glutinosa* L. (C. van Dijk)

Introduction

The density of nodules on alder roots is dependent on the nodulation rate and the mean life span. The nodulation rate in turn depends on the number of infective endophytic hypha strands and spores reaching susceptible parts of the roots per period of time and on the efficiency of the infection and nodulation processes. Thus, the density of the extra-nodular endophyte population (e.n.e. population) will to some degree determine the root nodule density in an alder population.

The e.n.e. population density is, in this particular case of an obligate symbiontic organism, mainly determined by the rate of influx from decaying root nodules and immigration, and by the rate of efflux caused by death of

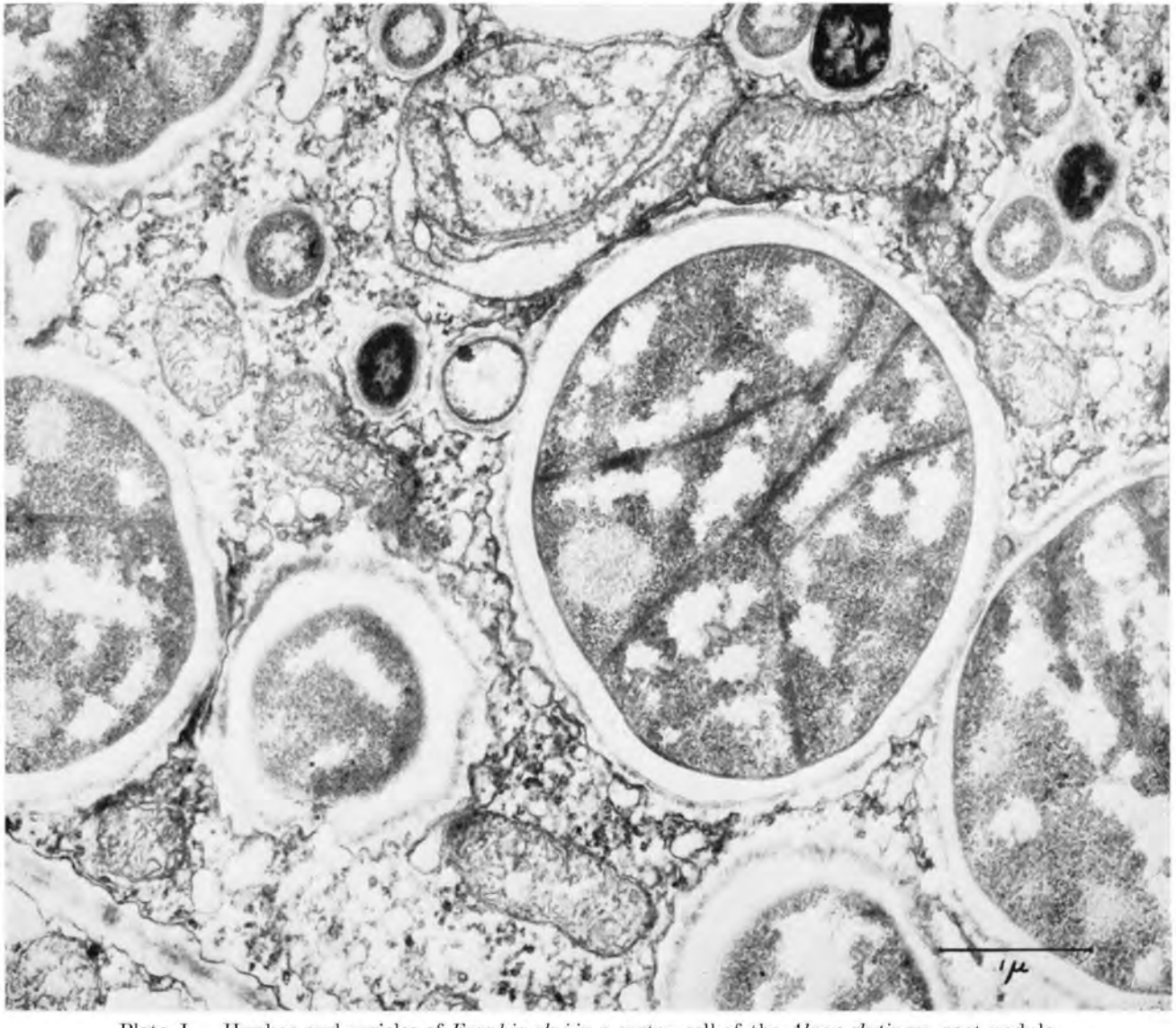

Plate I – Hyphae and vesicles of *Frankia alni* in a cortex cell of the *Alnus glutinosa* root nodule.

the extra-nodular endophyte and emigration. In earlier investigations (Akkermans & Van Dijk 1975) the annual influx of endophytic particles into the soil from decaying root nodules was calculated from the root-nodule turnover as estimated in an alder vegetation and from the experimentally estimated infective capacity per weight of living nodules. In these studies, which were restricted to spore-free nodules, a minimum influx rate of some 2,000 infective particles per gram dry weight of soil per annum was calculated. Analysis of the infection capacity of soil samples in the area under study indicated the presence of a minimum of 350–1,000 infective particles per gram of dry soil. These results suggest that the actual e.n.e. population density may depend entirely on influx from decaying nodules, without a contribution from extra-nodular growth. Unfortunately, no data on the death rate of the extra-nodular endophyte under natural circumstances are available for the area under study. It is highly probable that the root-nodule endophyte can survive outside the root nodule for periods of up to several years, but data on the death rate are only available from experiments carried out under unnatural conditions (Rogers & Wollum 1974).

Clumps of granule-shaped cells are often found within the root-nodule tissue. Microscopical investigation showed that these cells represent a stage in the life-cycle of the endophyte which is comparable to the arthrospores of free-living actinomycetes (Van Dijk & Merkus 1976). One characteristic, apart from morphological features, distinguishing the spore stage from the hyphae and vesicles, is that spores may be either present or absent within the nodule tissue. The ecological implications of the existence of spore-rich (Sp(+)) and spore-free (Sp(−)) nodules is not very well understood. From earlier nodulation experiments it was concluded that the absence or presence of spores in nodules represents a fairly stable strain difference rather than a purely phenotypic expression, as suggested by other authors (Schaede 1933). It was also found that the infective capacity of spore-rich nodules is about 500 times higher than that of spore-free nodules. This difference can be expected to bring about an equivalent difference in the rate of influx of endophytic particles from decaying root nodules into the e.n.e. populations of both strains. To explain the co-existence of these two strains of the endophyte, the supposed difference in influx rate must be counterbalanced by another strain difference. It can be questioned whether the strain difference evolved into an ecologically significant character enabling a wider range of host-endophyte interactions under different ecological circumstances or is counterbalanced by, for example, a high death rate of the liberated spores, in which case the ecological significance of the strain difference seems negligible.

Studies on the distribution of both nodule types in the field showed that coexistence of both types of root nodules at short distances, i.e., where the extra-nodular populations of both endophyte strains can be expected to compete for the same root surface, is a rather common feature. On the other hand, in some cases large areas (up to 1,000 m^2) were found to contain

only spore-free nodules (Van Dijk 1973). The occasional cases of considerable spatial separation between the two nodule types suggest that the strains behave differently with respect to at least one still-unknown environmental factor. Close coexistence of both nodule types might mean that the presence of microniches for each of the strains eventually determines the ratio of spore-free and spore-rich root nodules in a mixed nodule population, thus balancing the difference in infective capacity between the two strains.

To obtain information on the ecological behaviour of both strains of the endophyte, the following investigations were carried out:

1. A detailed description of the alder vegetation of a 140-ha dune area, followed by analysis of the distribution of both types of root nodules.
2. A comparison of the behaviour of both spore-free and spore-rich populations of the endophyte during and after root-nodule decay in soil samples collected from spore-free and spore-rich nodule areas.

Mapping of alder vegetations and rood-nodule types in a coastal dune area.

The area under investigation is a part of the dune region of Voorne and comprises 140 ha of young and old dunes managed as a nature reserve. About two-thirds of the area consists of rather natural dunes, dune ridges covered with shrub alternating with valleys. In this younger part of the area the valleys are covered with natural grassland and shrub vegetations; locally, the alder is present. The oldest part, covering about one-third of the total area, is influenced by small-scale agriculture. In this area small fields and waste land form an irregular mosaic pattern with a gradual transition to the younger uncultivated area. Almost all of the fields are surrounded by alder hedges, and small populations of alder trees are thought to have developed spontaneously on abandoned arable lands from seeds of these surrounding alder hedges. In other cases small groups of alder trees have been planted.

The area was mapped on a scale of 1:2000 by means of air photos and terrain reconnaissance. The alder trees were mapped, and their habitus, origin, and environmental circumstances were recorded. In addition, 42 stands in which alder was present were described in detail with respect to soil profile, ground-water level, and vegetation.

All soil profiles contain an A-horizont 3–15 cm thick, which is situated on the mineral C-horizont. Ground-water level varies from 0 to 50 cm below soil surface. The vegetation records permit the distinction of three types of alder vegetations, which show good correlation with the ground-water level.

1. Alder with *Stellaria media* and *Galium aparine*
   Maximum ground-water level −30/−50 cm.
   Variants wit *Urtica dioica* are present under alder hedges and in some younger alder vegetations.

2. Alder with *Moehringia trinervia*, *Listera ovata*, and *Eupatorium cannabinum*. Within this group 4 variants are distinguished, including dry variants with *M. trinervia* and *L. ovata*: ground-water level comparable to group 1 but older stands with a probably lower nutrient status of the soil; variant *M. trinervia* dominant with *Ajuga reptans*: maximum ground-water level −16/−20 cm; variant *M. trinervia* with *E. cannabinum*: maximum ground-water level −4/−14 cm.
3. Alder with *Hydrocotyle vulgaris* and *Scutellaria galericulata*
This group had highest maximum ground-water level (−3/+6 cm). The wettest alder stands of the area were present in this group.

Alder mapping and descriptions of the main environmental characteristics were used to set up a nodule sampling programme, the aim being to localize environmental factors affecting the distribution of both nodule types in the field. Nodule sampling is carried out at different locations along alder hedges and groves. At each location, nodules are collected at 3–5 regularly chosen sampling sites of 1 m^2 each. The results for some locations are already available. The results of the nodule analyses done so far show that Sp(+) nodules and Sp(−) nodules are found along the hedges. In most cases one nodule type dominates strongly per location or only one of the two nodule types was recorded. The few records collected for alder groves in the younger dune area all show spore-free nodules, but confirmation from additional nodule sampling is necessary.

Population dynamics of the endophyte during root-nodule decay under controlled conditions.

The influx of endophytic particles from decaying Sp(+) and Sp(−) nodules into the e.n.e. population of different soil types can be compared by quantitative endophyte analysis of decaying nodule and surrounding soil at different times after the onset of nodule decay. For this purpose, 4 sets of the following nodule-soil combinations were prepared:

1. 900 mg fresh Sp(−) nodule-lobes placed in 200 gr Sp(−) soil
2. 900 mg fresh Sp(+) nodule-lobes placed in 200 gr Sp(−) soil
3. 900 mg fresh Sp(+) nodule-lobes placed in 200 gr Sp(+) soil
4. 900 mg fresh Sp(−) nodule-lobes placed in 200 gr Sp(+) soil

Sp(+) soil was collected from an alder vegetation on river clay in which only spore-rich nodules were found. Sp(−) soil was collected from a dune area in which only spore-free nodules were present. For both soil types, the top 10 cm was collected after removal of the litter. The water content of the soils during incubation was 48% of the fresh weight for Sp(−) soil and 30% of the fresh weight for Sp(+) soil. This difference in soil-water content was introduced to maintain equivalent aeration in both types of soil. Each of the four combinations was prepared in 20 replicates in 250 ml jars. The nodule lobes were packed in nylon netting (30 mesh) together

with 3 grams of soil. These small baskets were placed in the centre of the soil. Samples were incubated in the dark. The temperature alternates between 10°C and 15°C at 12-hour intervals.

The infective capacity of the contents of the nylon netting and of the surrounding soil was determined at various stages of nodule decay. Details will be published elsewhere. Some preliminary results are given here. After 100 days of incubation the infective capacity of both types of soil surrounding decaying Sp(+) nodules (series 2 and 3) had been increased 100 to 1000 fold, where as that of the soils surrounding (Sp(−) nodules had increased 2 to 12 fold. These results show that Sp(+) nodules give 100 to 1000 times higher influx of infective particles into the e.n.e. population after nodule decay as compared with Sp(−) nodules. At this stage of the experiments there is little evidence that the influx rate or population density is affected differently by the two soil types. There is a slight indication that in Sp(+) soil a slightly higher density of the e.n.e. population is maintained after nodule decay than in Sp(−) soil. This applies to both nodule types. Incubation times longer than 250 days are still under study.

Myco-nodules

The nodulation tests in which an extract of Sp(+) soil was used as inoculum led to the formation of young root nodules which appeared to be initiated by a fungus instead of by the expected root-nodule actinomycete *Frankia alni*. In young stages these myco-nodules can only be distinguished from actinomycete nodules by microscopical observation of the nodule content.

The cortex cells are filled with thick hyphae. Zones of brown cortex cells are present at the periphery of the infected regions. After prolonged nodule growth, the difference between myco-nodules and actinomycete-nodules increases due to growth differences. Myco-nodules were found to grow very slowly and cease at a diameter of about 2 mm, whereas actinomycete-nodules continue to grow. We were able to induce myco-nodulation on alder with a suspension of crushed myco-nodules. One fungus, isolated from surface-sterilized, crushed myco-nodules on Czapeck-Dox agar induced myco-nodules on alder plants cultured on a Hoagland solution without nitrogen.

Identification of the fungus is in progress. The structure of the myco-nodules is in accordance with Pommer's (1956) description of myco-nodules on alder.

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LIMNOLOGICAL INSTITUTE - NIEUWERSLUIS

Progress Report 1975

History and function of the institute

The Limnological Institute was founded as a hydrobiological institute and was given its present name in 1968. Research is done on the biology and chemistry of fresh water oecosystems and its components.

Starting with one building at Nieuwersluis, a small village between Amsterdam and Utrecht, two new laboratories were build. The one in Nieuwersluis was finished in 1970 and in 1975 a second laboratory at Oosterzee on the border of Tjeukemeer was build.

An isolated sandpit (Lake Vechten) and lakes near the river Vecht are the field objects in the centre of the country, near Nieuwersluis.

In the northern part of the country, research on Lake Tjeukemeer was started as part of the International Biological Programme (IBP). IBP meetings were organized by the institute in 1966 and 1972. The work on Tjeukemeer was extended to cover the lakes of the Frisian polder system, of which Tjeukemeer is a part.

The institute also organizes courses and other training facilities in limnology for students of the universities. Training and research facilities are also offered to scientists from abroad.

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INTRODUCTION

Apart from the continuation of the research on the main objects of the institute, work was done on the phosphate budget of the Border Lakes of the Flevo Polders (part of the old IJsselmeer) and on the effect of heat disposal by an electrical power plant into the Bergumermeer.

Projectgroup Tjeukemeer

ABIOTIC ENVIRONMENT AND PRIMARY PRODUCTION (Dr. H. L. Golterman)

The results of six years' study will be used for a computation of primary production as controlled by the surface irradiance, light attenuation and the supply of nutrients, in particular nitrogen and phosphate.

The dilution rate of the lake is computed by using a modification of Biffi's formula.

In the primary production measurements, a striking difference was found between the gross and net production rates. There were sharp peaks in the oxygen consumption rates in April and August, not related to hydrological changes. The peaks in April coincided with high ammonia concentrations of which, however, no data are available for August.

The chlorophyll concentrations were relatively low during the whole year and do not reflect any influence of the abnormally high temperatures. Very low values were found in July, accompanied by low photosynthesis values. In general there is a good correlation between chlorophyll concentrations and photosynthesis values.

When comparing the chlorophyll and net primary production values of 1971/1972 and 1975, it is apparent that though the chlorophyll values are similar, the production values are lower in 1975. Part of this decrease in production might be due to an increase in the oxygen uptake rates, which could have been caused by the higher temperatures.

In order to examine the possible role of nutrient limitation in algal growth, bioassay studies were carried out at irradiance values approximating I_k. The results obtained so far are not yet consistent. In some instances nitrogen enrichment stimulated the algal growth rates, but in other instances enrichment with phosphate gave an increase of the growth rate.

Chlorophyll concentration does not appear to be a satisfactory parameter for algal biomass, as even in short term experiments adaptations to the changed environmental conditions occur. Recycling of nutrients in the dark water layers and in the bottom have not been taken into account in the experiments mentioned. Therefore, measurements using the bioassay *in situ* have been started.

BACTERIOLOGICAL AND ECOLOGICAL IMPORTANCE OF HUMIC SUBSTANCES (Dr. H. de Haan)

In conjunction with the work on the humic substances of Tjeukemeer, cumulating in his thesis: "Limnologische aspecten van humusverbindingen in het Tjeukemeer", de Haan investigated the composition of fulvic acids in the epilimnion and the hypolimnion of a sandpit near Oosterzee.

To study the effects of fulvic acids and sugars in natural waters, methods are being developed to separate amino acids and fulvic acids eluted simultaneously from a Sephadex column, and improvement of the analysis of sugar in fulvic acid fractions.

At the end of August de Haan left for a one year's period of study of organic carbon substances in Lake Saimaa (Finland).

SEASONAL PERIODICITY OF PHYTOPLANKTON (Mrs. Drs. M. A. C. I. Blaauboer-Wiercx, Dr. J. Moed)

Mrs. Blaauboer continued her investigation of the influence of environ-

mental factors on the growth rate of some Chlorococcales. Enrichment experiments with *Scenedesmus quadricauda* and *Monoraphidium setiforme* in water from Tjeukemeer gave results supporting the idea that growth in this lake might be nitrogen-limited in summer. The relation between the irradiance and the specific growth rate of *S. quadricauda* were determined in batch-cultures; an irradiance of 4–8 W/m^2 (on the inner surface of the culture flask) appeared to be saturating. Further work is done in preparation of chemostat experiments under conditions of nutrient limitation.

The periodicity of *Diatoma elongatum* in Tjeukemeer was further investigated by Moed. As silicate in early spring is determining the size of the populations of diatoms in this lake, experiments were started on the source of silicate.

Preliminary results indicate that the concentration in the lake is lower (ca. 4.5 mg Si/l) than in the waterways leading to the lake (7–7.5 mg Si/l). Out of mud from the lake, silicate was liberated and afterwards the mud could take up an amount of silicate again. This phenomenon was not observed when peat from the lake was used in the experiments. It is likely that clay plays an important role in the absorbtion and release of silicate.

Culturing *D. elongatum* in media not containing silicate led to the development of bi-protoplasmatic cells that fail to form a new cell wall.

POPULATION DYNAMICS AND PRODUCTION OF ZOOPLANKTON (Drs. J. Vijverberg)

Apart from the work on the population dynamics and production of copepods and cladocerans of the open water of Tjeukemeer, Vijverberg did some observations on the zooplankton of the littoral zone. The seasonal periodicity in the occurence of each species was similar in the three years of sampling (see also Progress Report 1972). Most copepod species were present during a large part of the year. Naupliar densities (not determined to species level) showed two maxima, one in May and the other one in July. All cyclopoid species went into diapause: *Acantocyclops robustus* (December-February) and *Mesocyclops leuckarti* (November-March) in winter, and the other abundant species in summer. Diapause occurs commonly in a particular copepodid stage, i.e. stage IV (*Diacyclops bicuspidatus, Cyclops vicinus vicinus* or stage V (*M. leuckarti*). In *A. robustus* the resting stages were copepodid stages III and IV in 1968/1969 and 1969/1970 but in the next two winters copepodid stages IV and V were found. This phenomenon could be due to early ice cover in the two earlier mentioned winters. A minor part of the population of M. leuckarti (1–6%) passed diapause in stage IV instead of stage V. A similar situation occurs in *C. vicinus vicinus* where 5 to 8% of the population remained in stage III. The length of the diapause period was different for each species: *D. bicuspidatus* (23–28 weeks), *M. leuckarti* (15–23 weeks), *C. vicinus vicinus* (7–10 weeks) and *A. robustus* (6 weeks). The percentage of adults, relative to the total

number of copepodids in these species populations are: 50.4% for *E. affinis*, 23.0% for *A. robustus*, 22.3% for *M. leuckarti*, 17.3% for *C. vicinus vicinus* and 5.0% for *D. bicuspidatus*. Although half of the sub-adults were ♀♀, the percentage of adult ♀♀ relative to the total number of adults was often lower than 50%. *A. robustus* (17–20%) showed the largest deviation from the expected value, which was smaller in other species. The differences in adult percentages and sex-ratio might be explained by differences in the size-selective predation pressure exercised by fish upon adult copepods.

PRODUCTION OF CHIRONOMIDS (D. M. Beattie M.Sc.)

Beattie continued his investigation of the chironomid larvae populations in Tjeukemeer and other Frisian lakes.

The over-wintering generations of 1974–1975 were present in all lakes. The larval densities ranged from c. 150/m² in the Leijen to c. 1500/m² in Sneekermeer. Two summer generations developed in 1975, with maximal larval densities of c. 400/m² in Heegermeer and Fluessen, while in all other lakes the densities were lower.

The relative occurrence of chironomid larvae of different species in the diet of the bream (*Abramis brama*) was analyzed. Data obtained so far indicate random feeding of the bream in the (complete) darkness of the Tjeukemeer bottom water. Further research will be done on this predator-prey relation, taking into account the morphological properties in the developing young fish and its specific behaviour.

GROWTH AND STOMACH CONTENTS ANALYSIS OF FISH (D. M. Beattie M.Sc., Drs. W. L. T. van Densen, Drs. J. Vijverberg)

To complete a study started in 1971, special attention was given to the population of older bream (*Abramis brama*) in Tjeukemeer. The growth was followed where age determinations were done by counting the annual rings in cross sections of fin rays. Satisfactory results were obtained using this method and the assumption of the existence of an exceptionally strong year class of 1970, based on length-frequency distribution could be affirmed. Growth appeared to be less than commonly mentioned in the literature, though more than recorded in the period until 1970 in Tjeukemeer. For a comparison of the growth, the population of bream in Langweerder Wielen was also sampled. Although food conditions were better in Langweerder Wielen (chironomid larvae), growth of the bream was similar to that in Tjeukemeer.

Work on smelt (*Osmerus eperlanus eperlanus*) was continued. Growth in Tjeukemeer seems to be highly dependent on the autumn occurrence of *Daphnia hyalina*; in some of the other lakes *Neomysis integer* was the most important food organism. Fecundity determinations in smelt (52–85 mm) from both Tjeukemeer and the Leijen made it possible to express the absolute fecundity as $4.69 \cdot 10^{-3} \cdot L^{3.068}$ (L in mm).

A study on the growth and production of 0+ fish in both littoral and open water was started.

THE FRISIAN LAKES (D. M. Beattie M.Sc., Drs. W. L. T. van Densen Dr. H. L. Golterman, Drs. J. Vijverberg)

From the results obtained during the previous five years by a routine sampling program, it seems that the Frisian lakes can be regarded as one entity. The phenomena occurring in one lake are recurrent in the other lakes. However, some biological specificity can be recognized to belong to one lake or a group of lakes within the Frisian lake system. When chlorophyll *a* in the lake water is taken as a measure of algal biomass production, an annual variation for the whole system is observed, occurring also in the individual lakes. However, there is a degree of variation common to a group of lakes or even to one lake only. The annual variation in the Leijen for instance, is low and does not correspond to the mean variation for the whole system, while having a high algal production. The Oudergaster Brekken and Grote Gaastmeer that have a high annual algal production as well, have an annual variation similar to that of the whole system. The production of algal biomass in the Heegermeer-Fluessen-Morra complex of lakes is lower than in the other lakes mentioned, but the annual variation is great (for a survey of the lakes in the Frisian polder system see Progress Report 1974).

Densities of cladoceran are comparatively low in the Frisian lakes, but the Leijen and the Heegermeer-Fluessen-Morra complex have high densities. In September smelt constitutes 50% of all 0+ fish in the Frisian lakes, except for the Leijen (62% bream) and the Slotermeer and Fluessen-complex (44% and 49% perch respectively). The *Chironomus plumosus* larval population density in the Leijen is very low compared to the other lakes. Further work will be done, to analyze the meaning of the dissimilarities found and to determine the importance of the geographical situation.

Projectgroup Lake Vechten

ABIOTIC ENVIRONMENT AND MUD-WATER RELATIONS (Drs. H. Verdouw)

The investigation of the factors regulating the concentrations of iron and manganese in Lake Vechten was continued.

The lake consists of two sandpits with a maximal depth of 12 m (W) and 10 m (E) separated by a ridge where the depth does not exceed 7 m. Most of the work has been done in the Eastern part of the lake. In 1975 detailed work was done in the Western part also, to compare the two parts of the lake. Differences in pH-value, alcalinity and manganese concentrations were observed. The manganese concentration was lower in the Western part (max. 5 $mg.l^{-1}$) than in the Eastern part (approx.

7 mg.l^{-1}). In both cases, however, laboratory experiments with artificial sediment/water systems indicated that carbonate formation was the factor regulating the manganese concentration.

For both iron and manganese, the amount of precipitating substance was determined using sediment-traps. The sediment-trap data indicate that daily 0.1-0.2% of both iron and manganese present in solution in the hypolimnion is precipitating. Less than 2% of the reducing power in the lake, generated by primary production, is calculated to appear in the iron (II) and manganese (II) fraction.

The present study of iron and manganese in Lake Vechten will be terminated and further work will be directed towards the investigation of the nitrogen cycle in close connection with work on the aerobic and anaerobic mineralization of organic material.

ECOLOGY OF ANAEROBIC BACTERIA (Dr. Th. E. Cappenberg)

In the investigation of the acetate fermenting *Methanobacterium* sp. and *Desulfovibrio desulfuricans*, a commensalism between the two species became apparent. Mixed continuous culture experiments showed that *Methanobacterium* benefits from the acetate released by *Desulfovibrio*; the latter not being influenced by the presence of the former.

The occurrence of the two bacteria at different depths in the mud, may be explained by the production of H_2S and acetate by the sulphate reducers in the upper layer of the mud. The methane producers being more sensitive to H_2S, will occur deeper in the mud. The redox potential is an additional factor responsible for this phenomenon.

The break-down of acetate was measured in experiments in which mud was incubated in Warburg vessels and the $^{14}CO_2$ and $^{14}CH_4$ produced from the 1-^{14}C-acetate, 2-^{14}C-acetate or U-^{14}C-acetate were trapped. The ratio of $^{14}CO_2/^{14}CH_4$ had a value of 1.32 in the case of U-^{14}C-acetate, indicating that 0.86 moles of CH_4 and 1.14 moles of CO_2 are formed per mole of acetate. Although the oxidation of the methyl carbon of acetate to CO_2 was quantitatively more important than the reduction of carboxyl carbon to CH_4 (32% and 13% respectively), both the methyl and carboxyl carbon were reduced to methane. The oxidation of the methyl carbon of acetate showed a dependence on the sulfate concentration in the mud samples. Further investigation of the turn-over rate constants of intermediate metabolites and the break-down in methanogenesis is being carried out. The methane might be oxidized by sulfate-reducing bacteria in this anaerobic mud.

Apart from these experiments, the production of methane under *in situ* conditions was assayed from mud samples in Warburg vessels connected to a Gilson respirometer. Assuming that the rate of methane production measured in this way is representative for the actual rate of production in the lake and only the upper 5 cm of the mud layer contributes to the production of methane, a calculation was made of the daily output of

carbon. As the measured production rate of methane under the conditions of the assay was 0.034 μmole per gram of wet mud per hour, the production in 1 ha of the lake bottom mud layer would amount to $5 \times 10^8 \times 0.034$ μmole = 17 mole of methane/ha of wet mud.hr^{-1}. Which means a production of 400 moles of methane per ha per day, corresponding to 4,800 g C/ha of wet mud per day. The input of carbon by primary production of phytoplankton in 1974 was 0.52 g C/m^2.day. As the photogenic zone of Lake Vechten has a depth of 6 m and a volume of approx. 214,000 m^3, the daily production in this zone will be $0.52/6 \times 214{,}000$ g C.day^{-1} = 17,120 g C.day^{-1}. There is also an input of carbon by the primary production of macrophytes and algae in the littoral zone, which is not quantified as yet. It is quite clear that anaerobic mineralization plays an important role in the carbon cycle of Lake Vechten.

PHYTOPLANKTON-ZOOPLANKTON ENERGY TRANSFORMATIONS (Dr. R. D. Gulati)

The studies on the feeding and metabolism of zooplankton in Lake Vechten were continued for the fourth year in succession. Moreover, in this year, the diurnal vertical migration of zooplankton and the accompanying changes in the feeding rhythm were also investigated.

The zooplankton had a mean standing crop of 0.5 g C.m^{-2} in winter, which increased significantly in May and the year's maximum value was recorded on 10 June as 1.9 g C.m^{-2}. More than 95% of the zooplankton biomass consisted of filter-feeders, with *Daphnia cucullata* and *Bosmina longirostris* as the dominant species. *Daphnia* sp. and *Diaphanosoma brachyurum* became abundant in August and dominated the third major peak of zooplankton on 2 September.

Suspended particulate organic matter that form the main food of filter-feeders (<15 μ) decreased from 4.0 g C.m^{-2} in spring to less than 1.0 g C.m^{-2} in the beginning of September. In the corresponding period this fraction of the total seston decreased from 90% to 15%. This continuous decrease in the level of food of the filter feeders during a period of high primary production (0.5 g C.m^{-2}.d^{-1}) in summer, is attributed to the grazing pressure of the zooplankton.

From the observations during the last four summers, it may be broadly generalized that the level of the food for the filter feeders in the lake vary between 2 and 3 g C.m^{-2} and the biomass of the filter feeders between 0.6 and 0.9 g C.m^{-2}. Approximately 12 to 27% of the food is daily grazed upon by the filter feeders, with ingestion rates ranging from 0.30 to 0.56 g C.m^{-2}.d^{-1}, amounting to 80 to 150% of the daily nanoplankton primary production. The assimilation efficiencies fluctuate between 30 and 60%.

The diurnal grazing rate data showed that intake was 2.5 to 4 times faster at dusk and dawn than during mid-day.

The various species of filter feeders showed preference for different depths in the lake. *Daphnia* sp. prefers 2.5–5 m depth and its interaction

with *Diaphanosoma* is strong. *Ceriodaphnia* sp. and *Bosmina* sp. prefer greater depths. *Diaptomus* sp. and *Diaphanosoma* sp. occur at various depths, but tend to concentrate in the upper layers at night.

Biochemical analysis of zooplankton shows that an appreciable fraction of their nitrogen is of non-protein nature. Fat content may reach a value of 24% in winter, whereas the mean value is 16%. There was a highly significant decrease in energy content of zooplankton from 6.6 cal.mg^{-1} DW in March to 4.9 cal.mg^{-1} DW in June ($P < 0.01$).

SEASONAL PERIODICITY IN ALGAE (Mrs. Drs. M. A. C. I. Blaauboer-Wierex, Drs. E. J. P. J. Mols and Miss M. D. Trommel)

The phytoplankton in Lake Vechten was dominated by *Chlorella* sp. and *Closteriopsis* sp. from November until March. During early spring Baccillariophyta were very abundant (*Asterionella*, *Stephanodiscus* and *Centronella*). Their numbers decreased in early summer and they were replaced by Chlorophyta (*Selenastrum*, *Tetraedron*, *Scenedesmus*, *Elakatotrix*, *Gemellicystis*, *Ankyra* and *Closteriopsis*) and Pyrrophyta (*Rhodomonas* and *Ceratium*). As was the case in previous years, again an algal layer was observed in summer at the oxygen zero line. It consisted of *Lyngbia* spp. mainly. Cyanophyta (*Meresmopedia*, *Oscillatoria*, *Lyngbia*, *Anabaena* and *Aphanizomenon*) and Euglenophyta (*Trachelomonas*) formed the greater part of the phytoplankton population in late summer. In autumn Baccilariophyta (*Asterionella* and *Synedra*), Pyrrophyta (*Rhodomonas* and *Cryptomonas*), Chlorophyta (*Elakatotrix*) and Chrysophyta (*Mallomonas*) build up the phytoplankton population. *Trachelomonas volvocina* and *Rhodomonas minuta* were common throughout the year. *Chlorella* cf. *pyrenoidosa* was also present during the whole year. Scenedesmus was observed in a two-cellular stage throughout the year, whereas in 1974 it was only observed in spring.

Fig. 1 gives a survey of the periodicity of some of the dominant algal species.

DEVELOPMENT AND MORPHOGENESIS OF ALGAE (Dr. C. L. M. Steenbergen)

Studies on the effect of nutrient concentration, temperature and light on the form of coenobia of *Scenedesmus* quadricauda was continued. In *Scenedesmus quadricauda*, a high degree of morphological variation occurs in batch cultures as well as in synchronized cultures. *S. quadricauda* produces unicellular stages, bearing two spines at each pole, and coenobia consisting of either 2, 4 or 8 cells, with two spines at the outermost cells. Using synchronized cultures, it was shown that the length of the photoperiod is decisive for the formation of either unicellular forms or coenobia. In relatively short photoperiods (e.g. LD: 6/18 hrs) only coenobia are formed, whereas long photoperiods (LD: 11/13 hrs) favour the formation of unicells.

The development of both forms was studied. The pattern of the first

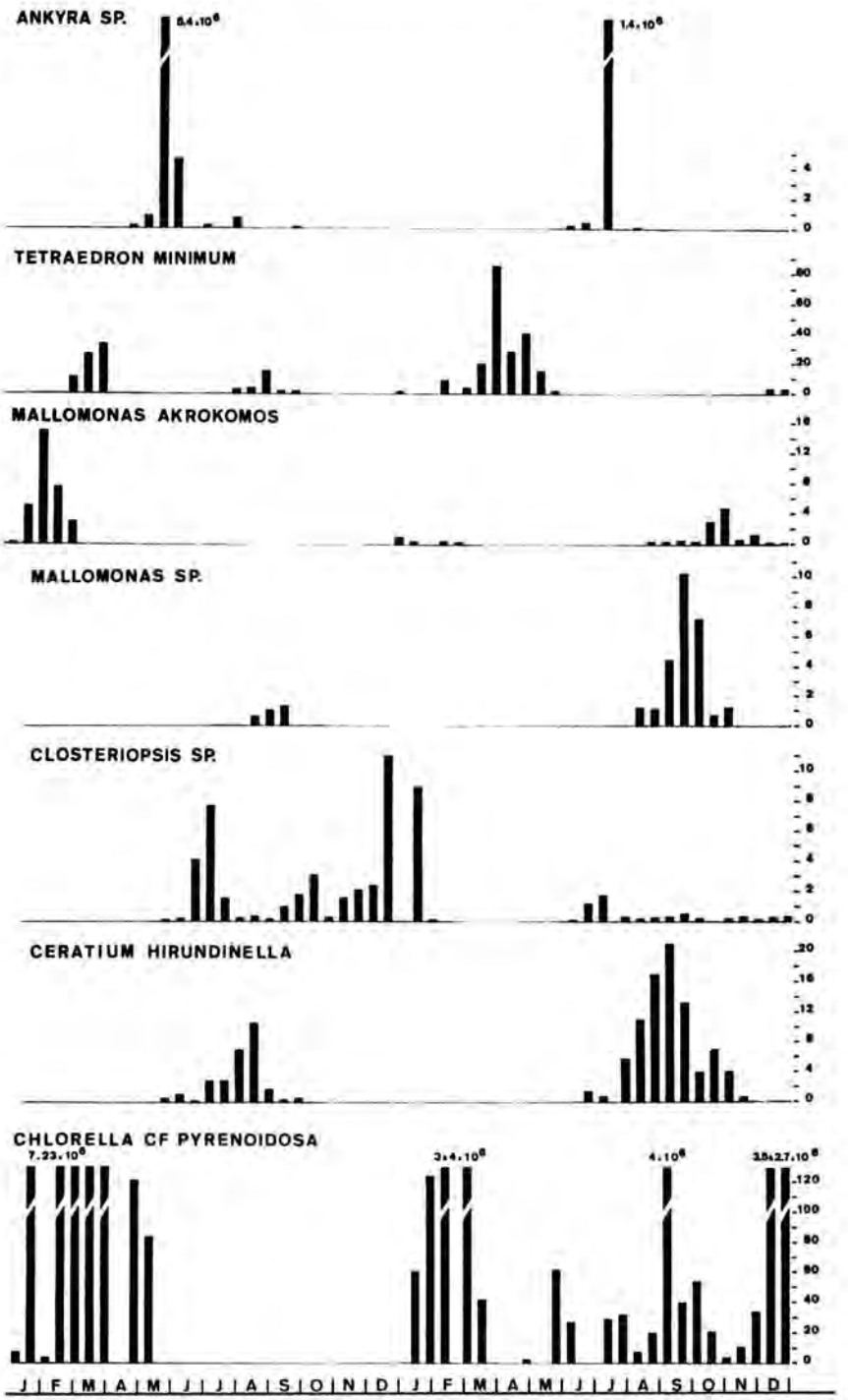

Fig. 1. Occurrence of some of the dominant algae in Lake Vechten.

and the second cleavage is similar in light-dark cycles yielding either unicells or coenobia. After the cytokinesis, the unicellular stages become ovoid in shape and are released from the parental wall as separate cells. The coenobial cells, on the other hand, elongate and adhere to each other.

In addition to this study of *Scenedesmus* species, which are the most common in summer plankton, the mode of reproduction and the process of encystment (formation of resting spores) in *Mallomonas akrokomos* (Chrysophyceae) are under investigation. *M. akrokomos* occurs in great numbers from November until April and may be regarded as a typical representative of winter plankton.

MORPHOGENESIS AND PHYSIOLOGY OF AQUATIC MACROPHYTES (Miss Drs. P. H. Best and Dr. R. Soekarjo)

For three years in succession, the morphogenesis of *Ceratophyllum demersum* and *Elodea canadensis* was studied. Seasonal changes in morphology and nutrient content was followed and recorded. The carbon, nitrogen, protein and starch content showed a seasonal dependency. Preceding and during vegetative growth in spring and during the formation of hibernacula (late spring and autumn), a high percentage of the nitrogen is bound in protein. Starch is accumulated in winter and metabolized in spring. The ATP content is high in summer and winter, but low in both the period in which the plant starts to grow in spring and during the formation of hibernacula. The sugar content is lower in summer than in winter, with no distinct maximal values. The calcium and manganese content were season dependent. No seasonal dependency was observed in the case of iron and magnesium.

Competition between *Ceratophyllum demersum* and *Elodea canadensis* was investigated. *Elodea* showed better growth than *Ceratophyllum*, when both species were grown together at a depth of 0.4 and 2.0 m. This was both the case when the species were already established in the experimental basin or not. The greatest increase in fresh weight occurred when grown at a depth of 2.0 m, in the case of both species. *Ceratophyllum* showed a better growth when anchored to the bottom, whereas *Elodea* starts faster when the shoots are buoyant.

The effect of exogenous IAA or GA on the morphogenesis of *Ceratophyllum* in the course of the year was studied under experimental conditions. IAA enhances the growth in length and the initiation of buds from December until April, in concentrations of 10^{-9} and 10^{-6} M, while 10^{-4} is a supraoptimal concentration for growth. IAA as well as GA do not cause an increase in bud growth. The growth of the buds occurs in April only and is not promoted by addition of either IAA or GA. There is a dose dependent increase of the stem length when GA is administered. The maximum effect of GA is obtained in December already, whereas the greatest effect of added IAA is observed later.

When ABA is given continuously, growth will stop. Given only at the

beginning of the experiment, growth will resume after a certain period of retardation. ABA stimulates starch and sugar production in a high concentration (10^{-5} M). It has however, little effect on protein synthesis. In low concentrations (10^{-9} M) it may cause a slight promotion of growth, concomitant with a similar or lower sugar and starch content than in the control plants.

Other projects

1. BORDER LAKES OF THE FLEVO POLDERS (Dr. H. L. Golterman)

The phosphate budget of this part of the former IJsselmeer was estimated in 1967 and 1971, showing a PO_4-P loading of about 2 to 3 $g.m^{-2}.y^{-1}$ with the sewage plants of Harderwijk (70%) and that of Elburg (10%) being the major sources. Since the lakes are 20 years old and because the phosphate content of the water leaving the lakes is negligible, about 50 $g.m^{-2}$ of PO_4-P is the estimated amount of phosphate accumulated in the bottom sediments since the formation of the lakes. The question now arises as to how rapidly the lakes can be restored after a diversion of 80% of its present phosphate load.

For the investigation of this problem, the Board of Zuiderzee Works of Rijkswaterstaat, has constructed an artificial pond similar to the so called „Lund tubes" in Blelham Tarn (England). Between 16th July and 22nd October the pond was flushed with polder water poor in phosphate and nitrogen. This resulted in a decrease of the chlorophyll *a* concentration by 50%. After nine days the pond was flushed with approx. 1.7 $g.m^{-3}$ of NO_3-N. The chlorophyll *a* concentration increased rapidly and almost reached the starting value. Although the pattern of changes in phosphate concentration were not as clear as those of chlorophyll *a*, there was a good correlation between the increase of chlorophyll *a* and that of Part-P. The increase in Tot-P after the fertilization was 1200 $mg.m^{-2}$.

During the experimental period, the concentration of NTA extractable sediment-phosphate decreased. The extraction was carried out with an improved method, where the sediments were mixed with (acid) washed hyflow cell and poured into a column. Extraction took place with 0.01 M Ca-NTA and subsequently with 0.01 M Na-NTA. The Ca-NTA extractable compounds decreased first, followed by a decrease of the Na-NTA extractable compounds. These compounds are considered to be hydroxy-iron-phosphate and apatite.

The decrease in PO_4-P from the pond sediments was 1000 to 1600 $mg.m^{-2}$. These figures correspond well with those of the gain in Part-P.

These results show also, that the bottom sediments act as a source of phosphate available for algal growth. Lake restoration will therefore be retarded by the presence of this phosphate source. However, the decrease of the concentrations observed after flushing, suggest that depletion may

be possible. A measure to this end could be the input of polderwater into the Border Lakes by the pumping-station „Lovink".

2. BERGUMERMEER (Drs. H. W. de Nie and Drs. J. B. W. Wanders)

As has been mentioned in last year's Progress Report, a study of the influence of heat disposal by an electrical power plant into the lake Bergumermeer was started. As the power plant was, however, not yet in full operation, and most of the water flowed in Northern direction out of the lake, not much effect on the phytoplankton species and their respective numbers was observed. The species occurring and their densities showed a large similarity to the situation in Tjeukemeer, so the latter lake can be regarded as a good reference, when further local rise in temperature will occur.

R. SOEKARJO

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DELTA INSTITUTE FOR HYDROBIOLOGICAL RESEARCH - YERSEKE

Progress Report 1975*

In 1957 the Division of Natural Sciences, reacting on an initiative of the Commission for Ecology, created an institute, to be established in the deltaic area of the south-west Netherlands, with the aim of studying the biological changes to be expected as results of the closing of the various river-mouths and sea-arms in this area.

When the Zuiderzee was closed by a dam and converted into the fresh Ysselmeer between 1920 and 1950, extensive biological research was carried out by a group of fishery biologists, members of botanical and zoological societies and academic staff, under the direction of Dr. H. C. Redeke. The important results obtained during this study, warranted the expectation that in the more diversified deltaic area of the rivers Rhine, Meuse and Scheldt, even more results could be achieved, especially so when one agency, located in the area, was given the task to make a co-ordinated effort to study the problems from various angles. After an exploratory phase, in which qualitative distribution of biota is to be studied from an ecological point of view, experimental work will be initiated, in order to elucidate the causal background of the changes observed.

The institute was erected under the name "Division Delta-Research of the Hydrobiological Institute", with the object to stress its affiliation to the Hydrobiological Institute at Nieuwersluis. As both institutes grew and matured the difference between the studies carried out in both of them gradually became apparent and in 1968 it was decided to change both names into their present form.

The institute is located at Yerseke on the Oosterschelde, the sea-arm to be closed in the last stage of the s.c. "Delta-plan".

The exploitation of the institute is financed by means of funds allotted to the Academy.

Scientific Staff (as per 31 December)

Dr. K. F. Vaas – Director
F. Vegter – Chemist
Dr. W. G. Beeftink – Botanist (Phanerogams)
Dr. P. H. Nienhuis – Algologist
R. Peelen – Planktonologist
C. Bakker – Planktonologist
Dr. S. Parma – Experimental Ecologist
Mrs. C. H. Borghouts – Zoologist
Dr. A. G. Vlasblom – Biomathematician
A. B. J. Sepers – Microbiologist
Miss. A. W. Stienstra – Experimental Botanist
Vacancy – Zoologist (from 1 October)

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Introduction

The institute has been created in order to study problems in the realm of aquatic oecology, the terrestrial oecological study of salt marshes and their biota also included. The institute was located in the centre of the deltaic area of the S.W. Netherlands, the area where drastic changes in flora and fauna were expected as a result of the realisation of the s.c. "Delta Plan", the closure of various sea-arms and estuaries by means of dams. During the initial, exploratory phase the inventory of the biota present and the analysis of their various reactions on the environmental changes involved, bore a more or less individualistic character. We have chosen now a multidisciplinary, oecological approach to a few central themes, using the drastic technical interferences of the Delta Plan as an opportunity to study oecological relationships operating within saline and brackish ecosystems. For this reason a different classification of the work carried out this year has been chosen for this annual report. The work done by the three working groups in operation now will be treated first, followed by a survey of the rest of our activities. These partly take the form of individual research, partly that of incidental observations in order to check long term trends studied previously. Some research activities started earlier, will be rounded off and finished.

Dr. W. J. Wolff left the institute in October in order to take up an important assignment elsewhere.

Two important additions to the equipment of the institute are worth mentioning. Our second research vessel "Maris Stella" was launched in October. The ship is equipped for pelagic fishing. Toward the end of December a green-house, located at the inner court of the institute, became available for culture and experimental work with halophytic plants.

On board the research vessel "Jan Verwey" appropriate facilities could be realised in order to apply for a license to work with radio-active material. This work will start in the first months of 1976.

Working group "Carbon cycle in the Grevelingen"

The central theme of this working group is a quantitative assessment of carbon- and energy cycling in the Grevelingen in its various hydrographic situations of a saline tidal basin, a saline stagnant basin and—eventually—a fresh stagnant basin. The latter situation is still uncertain as the fate of the Grevelingen is closely connected to that of the Oosterschelde and no decision as to the ultimate fate of this sea-arm has been reached to date.

In the Grevelingen it is our intention to study the influence of the changed environmental circumstances on the ecology of the basin from the functional point of view. To this end we try to study as many different components of the energy chains in a qualitative as well as in a quantitative way. The study of the situation previous to the building of the secondary dam at Bruinisse—closing the basin on the landward side—is based on

analyses carried out in the period 1964–1970. At the moment intensive research is carried out during the stagnant, saline period, lasting from 1970 to about 1980. As stated before the situation after that date remains uncertain.

Members of the working group are the staff members C. Bakker, P. H. Nienhuis, R. Peelen, A. B. J. Sepers, K. F. Vaas, F. Vegter and until October W. J. Wolff. External members are Miss T. Meijs (Agricultural University, Wageningen), H. G. Mulder (hydrographer), J. C. H. Peeters (Department of Roads and Waterways), B. Steinmetz (Fisheries Inspection) and K. Willems (State University, Ghent, Belgium), Nienhuis succeeded Wolff as team-leader. Vlasblom will assist in statistical matters.

This year again Sepers studied the role of dissolved organic substances in the water of the Grevelingen. The results obtained so far and shown in the following table, clearly indicate the importance of this item of the carbon cycle. Next year research into this matter will be intensified with the aid of our own equipment.

	Dissolved organic matter in ppm
December 1974	3
May 1975	6
August 1975	10–15
December 1975	15–20

Hydrochemically the basin can, at the moment, be characterised by the following data, according to Vegter's measurements: Salinity did not rise above 13.8‰ Cl (24.9‰ S). Nutrients reached slightly higher values than in 1974: with maxima of 27 μgat PO_4-P, 30 μgat NH_3-N and 25 μgat NO_3-N/l in winter, against minima of 5 μgat PO_4-P, 3 μgat NH_3-N and 0 μgat NO_3-N/l.

In a paper presented to the 10th European Marine Biology Symposium at Oostende (Belgium), Vegter treated phytoplankton primary production in the basin in relation to nutrient cycles during the period previous to the closure. His conclusion was that at that time the open Grevelingen estuary acted as a nutrient trap for organic nitrogen imported from the coastal water. By way of the primary production nitrogen from outside the system was incorporated into the estuarine ecosystem.

Miss T. Meijs (Wageningen) isolated and cultured 11 diatoms from the Grevelingen and started to make them free of bacteria. She also compared different methods for assessment of biomass of algae and finally adopted the measurement of total carbon content, this being the most reliable method. Another advantage is the small amount of material needed, about some milliliters, in which amounts 5 ppm can be reliably measured.

When Wolff left the institute in September, Nienhuis took his place

as team-leader and therefore changed the main point of his activities from synecological research of structure and dynamics of benthic algal vegetation to research on the processes of primary productivity of phytobenthos in the Grevelingen. Much of his time in the second part of the year and that of his assistent de Bree was devoted to preparatory work of this kind. Research in the basin will start in the first months of next year.

In the summer the dispersion of macro-algae and seagrasses was reinvestigated in order to make a comparison with the situation in 1973. The standing crop of the macrophytobenthos in summer proved to be increased by about 30%. About 70% of the total biomass was formed by seagrasses as shown in the following table.

	1973	1975
Cover of seagrass in ha (> 5%)	1585	2764
Ash-free dry weight seagrass (g/m^2) in relevant area's	130	98
Ash-free dry weight seagrass (g/m^2) total basin	19	25
Ash-free dry weight macro-algae (g/m^2) total basin	8	10

During the first years after the closure *Zostera* was only found growing on sand rich in silt, but now seagrasses have extended their territory to sandy soils also and are even growing between boulders. Owing to the large transparancy of the water seagrass is encountered even at a depth of 7 m, although the maximum biomass is found between 0.5 and 2.5 m.

Data on annual production of seagrass are being worked out. Brown and red algae, occurring two years ago with an ash-free dry weight of 5 g/m^2, decreased this year to 2 g/m^2, whereas green algae are increasing. The biomass of *Chaetomorpha* increased in two years from 0.8 to 5.8 g/m^2 ash-free dry weight.

Wolff summarized his studies on the open Grevelingen in three papers shortly to appear in print. In one of them an annual food budget for the zoobenthos of the still open estuarine Grevelingen is presented. In another paper special attention is drawn to the trophic role of birds during the estuarine phase, together with a summary of the changes in the avifauna after complete closure. In the estuary ducks, waders and gulls were the major species, subsisting mainly on benthic flora and fauna, in the saline, stagnant lake fisheating birds, e.g. grebes, cormorants and mergansers, came to the fore and waders declined drastically.

Concomittant changes in consumption were very pronounced. In the estuary 3.4 g ash-free dry weight per m^2/year was consumed from the benthic fauna, against 1.0 g/m^2 in the saline lake. Herbivore consumption rose from 0.1 g to 2.7 g ash-free dry weight/m^2/year.

As pointed out in the principal article primary production *in situ* and input of organic detritus from the coastal sea appear to be the most important food sources for the estuary. Input of detritus from salt marshes or other territorial systems seems to be relatively unimportant, a situation quite different from that encountered in American estuaries. In the Grevelingen tidal currents and the shallow depths of the basin together make phytoplankton production more readily available as food for zoobenthos than in deep estuaries where zooplankton will be more favoured. Consumption of phytoplankton by zoobenthos without interference of zooplankton, presupposes a vertical concentration gradient in phytoplankton. According to H. G. Mulder, such a gradient might be established and maintained via the combined action of sinking, turbulent diffusion and consumption by the zoobenthos.

This year research on meiofauna was started by K. Willems, assisted by A. J. J. Sandee. As hardly any data were available, this years research is a first exploration, based on the study of fortnightly samples collected by divers. Harpacticides and nematods proved to be the principle components. 49 genera of nematods were found thus far at an average density of 853.000 individuals/m^2. *Prochromadorella ditlevseni* is the main species, living in numbers in the upper 10 cm. *Theristus problematicus*, *Microlaimus sp.*, *Anticoma sp.*, *Enoploides cephalophorus* and *Monhystera sp.* must be mentioned among the nematods of the upper layers of the soil. *Sabatieria sp.*, *Theristus sp.*, and *Cabbia sp.*, on the other hand, were found to favour greater depths. Others, like *Neochromadora poecilosoma*, *Neochromadora poecilosomoides* and *Monoposthia spp.*, can be found equally numerous in all layers down to about 20 cm.

Five species of harpacticides were found so far: *Canuella perplexa*, *Asellopis hispida*, *Tisbe sp.*, *Paraleptastacus espinulatus* and *Stenocaris minuta*. The first three species only use the upper 2 cm of the bottom, the latter two are interstitial organisms living in the upper 10 cm zone and penetrating further downward to about a depth of 20 cm. Their average density amounts to about 445.000/m^2.

Vaas and P. de Koeijer continued their monthly sampling of benthic fishes. The population of plaice is aging, grows and decreases owing to the activities of numerous sportfishermen.

Diversity of the bottom fish fauna declines slowly, as, in spite of incidental finds of species caught occasionally and in small numbers, the number of aspect-forming species declines. No plaice smaller than 15 cm were caught and no other signs of reproduction were found. In winter 232 plaice captured during two fishing trips were aged by means of the otoliths. A total of 2.6% of them belonged to the year classes 1972 and 1973 and must have entered after the closure through the locks at Bruinisse. Females proved to be older than males. As expected, about 80% belonged to the year class 1971, those plaice that were trapped as one-year old animals at the moment of the closure.

Working group "Structure and Dynamics of the Saltmarsh ecosystem"

This working group was created in the course of this year and consists of Beeftink and Miss Stienstra and their assistants, with part-time assistance of Nienhuis and cooperation of Vlasblom as regards statistical matters.

This group has in view the study of structural and functional aspects of systems and subsystems in salt marshes and related ecosystems, with special emphasis on the influence of instability factors, such as the technical works of the Delta Plan, but also the construction of polders in former times. Work will be carried out on the level of associations as well as on the level of populations. Problems on the population level emmanate from previous studies on the dynamics of vegetations and the work aims at further analysis of the functioning of indicator species in the ecosystem. The concepts of demography and strategy are considered essential. Demography has a bearing on the actual behaviour of a population in the field, evaluated with the aid of quantitative data on e.g. life span, seed production, dispersal of seed etc. Strategy aims at the elucidation of the genetic potentialities of the species in relation to its survival under the ambient environmental conditions.

This year Mrs. K. van Noordwijk (Utrecht) finished her mathematical treatment of Beeftinks relevée's of the vegetation on the Middelplaten in Lake Veere, carried out under supervision of Miss P. Hogeweg (Utrecht). Cluster analysis and principal component analysis were used in order to study the spatial and temporal changes in the pattern of the vegetation caused by the closure of this former sea arm in 1962. In the first years the pattern is mainly a temporal one, in later years mainly a spatial one. Periods of rapid and slow changes were found to alternate in the pattern of the vegetation and clusters of mainly halophytic plants could be distinguished from clusters of glycophytes. These were characterized using the Kruskal Wallis index. In most permanent squares the number of species and the diversity increases until about the 7th year and decreases afterwards. However, in some squares diversity is still increasing.

The development of vegetation on recently permanently emerged sand flats around the Grevelingen, is similar to that on the Middelplaten, but in this case clearly less rapid. The reason might be sought in a lower quantity of silt in the soil and thus a greater danger of sand being blown away by the winds. As these terrains are much larger the supply of diaspores is bound to be less than on the Middelplaten.

Changes in vegetation along the Volkerak, where the tidal amplitude was raised, were studied this year again. In the zone where a rise of about 40 cm took place, these changes were most conspicuous. As a reaction on the sudden rise of the water level an immediate process of deterioration took place and the vegetation died in many places. Afterwards recolonisation set in and a stable situation is now seen to develop gradually. The ultimate

situation is one where a vegetation type is found similar to the ones growing before the rise of the water on a lower level.

A final report on the studies J. Leemans and B. Verspaandonk (Nijmegen) had carried out in 1971–1972 on the vegetation of Saaftinge, under supervision of Beeftink, became available this year.

The characteristic features of this brackish salt-marsh vegetation are outlined. Aerial photographs were used to draw a vegetation map (1 : 10.000), to detect pedo-genetic progress and to study the influence of grazing. This report was much valued in circles of nature conservationists, emphasizing the importance of Saaftinge in relation to the plans for the digging of a canal at Baalhoek and the normalisation of the Western Scheldt, which plan might endanger the area.

In June Nienhuis defended his thesis on biosystematics and ecology of the green benthic alga *Rhizoclonium riparium* at the University of Groningen. The work has been carried out between 1967 and 1974 and describes the ecological role of this alga in the border zone between land and water. The influence of various environmental factors on morphology and reproduction is described and the extremely wide ecological scope of the species, growing from the sublittoral zone on to the zone reached during storms, is underlined.

The student H. C. Mennes (Utrecht), who evaluated different sampling techniques of benthic algae on salt marshes under supervision of Nienhuis and Miss P. Hogeweg (Utrecht), finished his report this year. For the s.c. "General Chlorophyceae community" – consisting of small green and blue-green algal species and showing a wide dispersion in our area – a minimum area of 35–50 mm^2 could be established. He tested the feasibility of different sociological estimates as well as the homogeneity of the algal mat, with the aid of cluster-analysis. The methods used for higher plants proved to be inadequate and have to be modified.

Miss Stienstra's work on the influence of tidal movement on growth and development of *Halimione portulacoides* can be related under three headings. In the first place the capacity of germination was studied. From October till December 1974 seed had been collected and the germination was tested this year on wet filter paper. Although a good deal of scattering in the data hampers interpretation, the following preliminary conclusions might be drawn. Seeds collected in October germinated after 20 days, seeds collected in November-December after 5 days. With a photoperiod of 8 hours darkness and 16 hours of light the percentage of germinated seeds is higher than in constant darkness. In the second place Miss Stienstra succeeded in growing seedlings of *Halimione* and began studying their tolerance against NaCl. The plant needs NaCl for proper development, as most *Chenopodiaceae* do, but high concentrations slow down the growth rate. In the lower concentrations the ratio shoot/root increased, but in salinities over 0.2 n this ratio was seen to fall again. In the third place *Halimione* from different places were collected and in the fluid pressed

out of the leaves, the sugar and NaCl contents were measured and the osmotic value determined. Plants from the weakly saline salt marsh Springersgors showed a lower salt content than those from Stroodorpe-polder, where soil salinity is high. Both samples contained about the same amount of sugar. These investigations are being continued.

Working group "Structure and Dynamics of instable Aquatic Ecosystems"

The central theme of this, recently established group, in which Parma, Mrs. Borghouts and Merks participate with assistance of Vlasblom in statistical matters, might be described as follows.

Unpredictable natural or technical environmental changes of sufficient magnitude will seriously interfere with the structural and functional development of an ecosystem. Usually the result will be a marked retrogression to a level further removed from the initial one, the more powerful the environmental impact had made itself felt. When tidal influence abruptly comes to an end the ecosystem of a former tidal basin will almost have to start a new development from stretch. The same will happen when a saline basin is turned into a fresh one. In the initial phase of redevelopment the rate of change will be rapid and will decrease later on, so this initial phase can only be studied for a limited period.

Small and shallow inland waters differ from large and deep ones as regards the unpredictable and wide fluctuation in environmental changes they are exposed to, as well as in their smaller buffer capacity against untoward impacts from outside. Such waters are constantly exposed to sudden and irratic changes in oxygen content, salinity and water regime etc. In fact some of them are apt to dry up completely from time to time. This instable situation means that a steady development is repeatedly interrupted and an opportunity is offered to study the initial phase of development over longer periods. For such small water bodies the initial phase will in fact be the permanent stage they have to cope with. Thus research will be directed to the environmental factors which determine this stage, to the biota which manage to survive under these circumstances and the mechanisms which mean survival value for them. Mainly brackish inland waters will be chosen as first objects of research.

In relation ship with research initiated in previous years research proceeded this year along the following lines.

Merks, Parma and Mrs. Borghouts, together with their assistants, carried out a fortnightly sampling program in order to elucidate nutrient contents, ionic balance and some physical properties of nine ditches, moats and drinking pits for cattle. To this end Merks had to modify existing analytical methods for determination of high sulphate contents in brackish water. In these highly saprobic water nutrient content is bound to be high, as evidenced by the following data: Si-25 mg/l, $P\text{-}PO_4$ in solution—11 mg/l, $N\text{-}NH_3$—18 mg/l. A BOD_5 value of 38 mg/l also

illustrates the saprobic condition. Some elements, such as Ca vary irratically, as was also the case for HCO_3 ions, others, such as Cl, PO_4 and NO_3 showed seasonal periodicity to a certain extent. Often a liniair relationship could be found between the osmotic value and the chloride contents (milliosmol/kg = 0.8 + 52.7 × ‰ Cl). In a number of slightly brackish inland waters of a salinity less than 9‰ Mg and K proved to be correlated with Cl and showed seasonal periodicity, Na however, is far less correlated with Cl and was often seen to fluctuate irregularly. Ca fluctuates often negatively correlated with K and Cl.

In April and July a 125 cm deep moat was studied during a period of 24 hours, taking samples every half hour on different places. In a layer of about 20 cm over the bottom a clear-cut microstratification of oxygen and NaCl was found. The oxygen content showed a diurnal periodicity at the surface – in the centre as well as along the shore – with all values well above saturation in April and from oversaturation till near saturation in July. Near the bottom the above mentioned stratification caused the oxygen contents to fall to values fluctuating from 60 to 0% at a depth of 10 cm over the bottom, even at a total depth of 75 cm only.

The student C. van de Boogerd (Utrecht) carried out a qualitative inventory of the macrobenthos and macronecton of a number of creeks. In collaboration with Miss P. Hogeweg (Utrecht) a classification analysis will be carried out and an effort will be made to correlate the various clusters with environmental factors such as salinity, morphometry of the basin etc.

Mrs. Borghouts has been engaged in work on dispersion and life-cycles of Mysids for many years. As some of these opossum shrimps may be seen as typical representatives of inhabitants of brackish water it was decided to continue this work within the framework of this working group. This year sampling has been carried out on Goeree-Overflakkee, Tholen and St. Phlipsland. *Neomysis integer* was the principal representative in this area as well. As a preliminary working hypothesis it might be stated that its dispersion is not only governed by salinity but also by form and morphometry of the basin. Accessibility also seems to be important. In water bodies with salinities over 11–12‰ Cl *Praunus flexuosus* was often encountered next to *Neomysis*.

Another group of biota prevalent in the water bodies studied by this working group are the chironomides. Research on dispersion in Walcheren was provisionally rounded off as all imagines have been reared and identified. *Chironomus halophilus* Kieff. and *C. salinarius* Kieff. proved to be the most frequent species, especially so in brackish inland water bodies, where also *Leptochironomous deribae* Ringe is found. In water of still lower salinity the following species were found, enumerated in a decreasing order of abundance: *C. annularius* Meig, *C. plumosus* L., *C. thummi*, subsp. *piger* Str., *Camptochironomus pallidivitatus* Malloch, *Procladius breviatus* Remmert, *Psectrotanypus varia* Fabr., *Trichocladius*

lucidus Staeg. and *Cricotopus sylvestris* Fabr. A few species are still to be identified.

Glyptotendipes barbipes Staeg. and *Procladius choreus* Meig. proved to be rather euryhaline forms, being found at different salinities.

Parma finished his previous studies on the population dynamics of a moat in the Adriaanpolder. The density of the most common species *Chironomus halophilus* remained at a steady level of about 500 larvae per m^2 during winter, about $^1/_{10}$ of the density reached in 1974. *C. salinarius* maintained its density of 40 per m^2 in the same period.

Specially directed research

Bacteriological research

Sepers, continuing his research on the substrate specificity of ammonifying bacteria, has tested 45 isolates till now. In the following table the isolates are classified as to the number of amino acids utilized as sole source of carbon, nitrogen and energy.

Number of amino acids utilized	Number of isolated as a percentage of total tested
1– 5	11
6–10	32
11–15	45
16–19	11

From the data given above, it follows that specificity as regards to amino acids is slight. At the moment sugars, fatty acids and hydroxyacids are being investigated. Preliminary results on about 15 isolates indicate a similar low specificity.

In order to establish the relation between specific growth rate and substrate concentration (Michaelis-Menten curve) the maximal growth rate had been measured in previous years in batch cultures using histidine isolates. This year maximal growth rates of strains isolated on glycine, leucine and asparagine acid were determined. Measurements of "μ-s curves" in continuous cultures with bacteria isolated on histidine were started this year. When ultimately the entire Michaelis-Menten curve has been established an insight into competition between ammonifying bacteria in their natural habitat will have been obtained.

In other experiments bacterial samples growing at natural substrate levels were used to measure respiration and incorporation of labeled carbon compounds. In 1974 recovery of the ^{14}C-labeled substrate could only be achieved up to 70–80%. Some attention was given this year to an effort to increase this percentage. In order to measure the incorporated labeled substances in a homogenous counting system, solubilizers were applied.

Recovery of the $^{14}CO_2$-fraction was determined using labeled bicarbonates. After addition of acid the free CO_2 was fixed with phenetylamine and in this way the label could be almost quantitatively recovered. In a subsequent experiment labeled amino acid was added to a culture and the partition of the label into the fractions of $^{14}CO_2$, bacteria and remaining substrate was measured after 2 hours of incubation. A total of 97% could be recovered. Next year this method will be applied to measure the mineralization rate in the natural environment.

Plankton research

The work of Bakker and his two assistants on the plankton of Lake Veere this year was carried out with the aid of a Schindler-Patalas plankton trap, which apparatus obviates the escape reactions often exhibited by zooplankton when suction pumps are used. Results will be used for a further analysis of similarities and differences between the zooplankton of Lake Veere and that of a region of the Westerschelde estuary, where salinity is the same but many other environmental factors differ. Two papers on this subject were published jointly with N. de Pauw (Ghent, Belgium). In these papers it was pointed out that zooplankton development was quite different in these two waters, owing to differences in food supply, water movement and water exchange.

In stagnant Lake Veere zooplankton subsists on the diversified and dense phytoplankton, which situation favours rotifers, polychaet larvae and several other groups, including copepods, mollusc larvae and protozoans.

In the estuarine, turbulent tidal water of the mesohaline stretch of the Westerschelde detritus is the only important food source for zooplankton. Although phytoplankton is rather dense in summer, small cells, essential as food for rotifers, are rare as well as disperse. Tidal exchange causes transport of organisms in seaward direction. Therefore the environment does not favour rotifers, although copepods seem to be successfully adapted.

Owing to a very rainy autumn and early winter 1974 and spring 1975, salinity of Lake Veere was lower than in the previous year and fluctuated between 7.5 and 10‰ Cl. Many polyhaline-marine species abounding in the lake in 1974, were unable to survive now.

In September chlorinity rose to 12‰ and marine diatoms such as *Rhizosolenia setigera*, *Nitzschia seriata* and mainly *Lithodesmium undulatum* bloomed.

In the Oosterschelde also a low salinity was noted in the first part of the year and temperature in spring was low as well. This caused a delay in the onset of algal blooms in spring. Only in May proper spring blooms developed.

Terrestrial botanical research

Under Beeftink's supervision, three students, Miss J. H. Beckers, H. M. N. A. Dankers and H. A. J. Tevonderen (Nijmegen) constructed vegetation maps of 4 transects on the salt marsh near Bergen op Zoom and compared their results with similar work carried out in 1964. Numerous changes could be discerned, probably mainly caused by a larger supply of silt owing to the construction of the Scheldt-Rhine Canal. Taxa more numerous than in 1964 were *Spartina*, *Puccinellia*, *Aster* and *Elytrigia*.

The students E. H. R. R. Lammens (Nijmegen) and M. J. van Eeden (Wageningen) studied transport of detritus to and from a salt marsh at Stroodorpepolder. In accordance with the hypothesis proposed by Wolff (p. 5) it seems that more detritus is transported from the Oosterschelde towards the salt marsh than in opposite direction. Most detritus is transported at high flood levels. The total quantity of flotsam deposition against the dyke face amounts to about 10% of the total biomass of the marsh vegetation.

The student R. H. Kemmers (Utrecht) finished his report on an investigation of vegetation and environment of some "inlagen" along the Oosterschelde. "Inlagen" are mainly, low-lying and saline areas enclosed by an outer and an inner dyke. The vegetation of the "inlagen" is generally composed of a limited number of species. Next to specific characteristics of the environment, heavy and irregular grazing must be held responsible for this feature. In his work measurements of seepage were used to study the influence of a possibly reduced tidal range of the Oosterschelde, the ensueing lowering in the groundwater level and reduction in salinity. An evaluation of the various "inlagen" from the view point of nature conservancy was made.

Zoological investigations

The difficulties encountered by Mrs. Borghouts in 1974 in distinguishing between juvenile *Pseudamnicola confusa* and *Potamopyrgus jenkinsi* could be solved this year, when a population of *P. jenkinsi* was discovered not mixed with *P. confusa*.

Mrs. Borghouts continued her three-monthly sampling of the littoral fauna of the Grevelingen and did not find many differences with the previous year. *Lepidochiton cinerea* became more rare and sponges – mainly *Ciona intestinalis* – were more numerous. *Littorina saxatilis* managed to survive. Among mysids *Praunus flexuosus* is still dominating and *Mesopodopsis slabberi* increased in numbers.

The littoral fauna of Lake Veere did not change. As had been done in 1972, Mrs. Borghouts this year once more studied settling of *Teredo* larvae and found it to be less heavy. On logs placed in the lake in 1973, hardly any shipworms were found and divers could hardly find any along the shore. The bottom fish fauna did not show any changes. It is interesting

to note that divers observed the black goby *(Gobius niger)* in the Grevelingen, although this species could not be caught during the numerous fishing tours.

In previous years W. Rozing had taken blood samples of plaice from the isolated populations in Lake Veere and Grevelingen and also from North Sea plaice and had measured chlorinity and osmotic value in the plasma of fish taken the year round. Vlasblom analyzed the data statistically and came to the conclusion that the osmotic value decreases with increasing length of the fish, and that the osmotic value of plaice of the same length is higher in North Sea plaice than in those from both lakes. However, plaice from Lake Veere, where salinity is less than in Lake Grevelingen, showed an osmotic value higher than those from the other lake, which difference is only caused by an increased concentration of non-electrolytes. The electrolyte contents were the same. Further statistical analysis will reveal whether seasonal variation in relation to ambient water temperature can be established.

Continuing her studies on dispersion of mysids, Mrs. Borghouts found in the eastern part of the Westerschelde *Neomysis integer* dominating and in the western part *Gasterosteus spinifer* and *Schistomysis kervilei*. In some *Gastrosaccus* from Wester- as well as Oosterschelde the parasitic isopod *Prodajus ostendensis* was encountered. In July a fisherman brought us a 75 cm long specimen of the Sun fish (*Mola mola* L.) caught off the mouth of the Westerschelde. In the aquarium three different wrasses brought in by fishermen, could be kept alive up till now: *Labrus turdus* L., *L. berggylta L.* and another one, tentatively identified as *Acantholabrus palloni* but this identification needs conformation by a study of the specimen when no longer alive.

Chemical and biological research concerning water pollution

As in previous years some attention was given to the possibilities of water pollution and its possible effects on littoral biota near the outlet of the sewer at Waarde. As in 1975 a series of measurements of physico-chemical parameters was carried out during a complete tidal period of 13 hours by Merks. Preliminarily it might be stated that the sewage is rapidly absorbed by the water of the Westerschelde and that at a distance of 0.5 to 1 km beyond the outlet no influence is observed.

Analysis of samples of the littoral fauna near the outlet is not yet completely finished. *Hydrobia ulvae* is the dominant organism and it seems that number and average length increased. The analysis will decide whether other influences than those from the sewage are involved.

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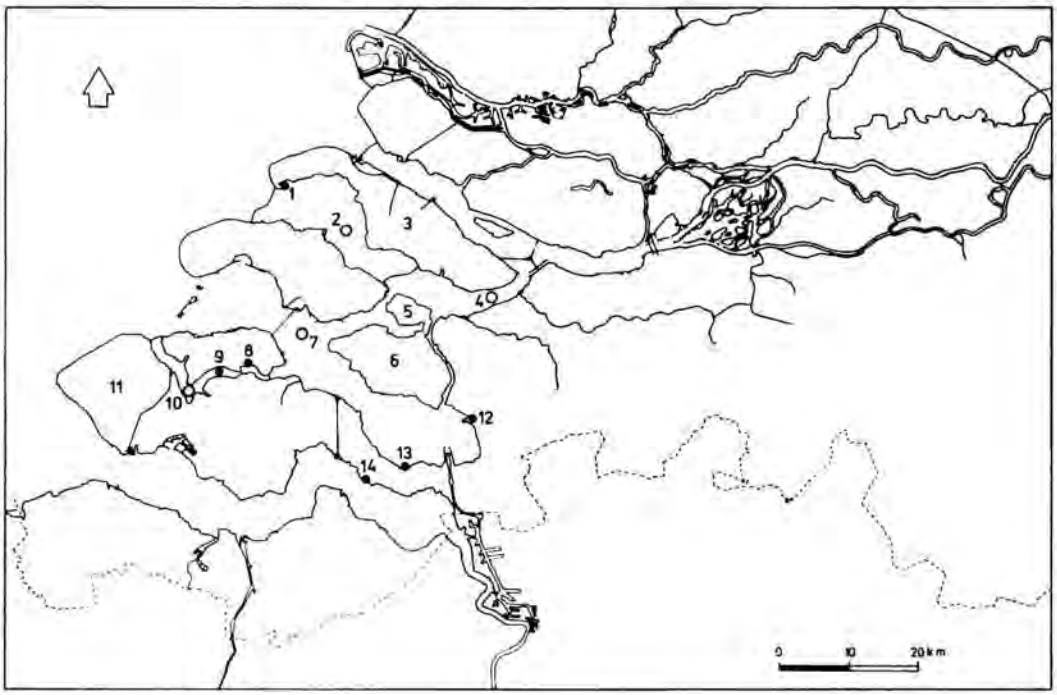

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