

Cytology of parthenogenesis in the tardigrade *Hypsibius dujardini*

DIETER AMMERMANN

University of Tübingen Zoological Institute

Received on September 22, 1967

Abstract. *Hypsibius dujardini* Dor. (*Articulata*, *Tardigrada*) shows obligatory parthenogenesis under given cultivating conditions. Males were never found. The first meiotic division reduces the number of chromosomes; the ($2n = 10$) chromosomes are divided between a small polar body and the egg nucleus. Prior to the second division the dyads divide, thus restoring the diploid number. A diploid polar body is formed subsequent to the second division. After the egg nucleus has moved toward the center of the egg, the cleavage divisions begin. — During meiosis II and the first cleavage divisions the chromosomes can develop into "large chromosomes" which presumably consist mostly of RNA. No "large chromosomes" are found after the seventh cleavage division. Sometimes a plate of coloured material ("elimination chromatin") can be observed between the anaphase daughter plates of the first cleavage divisions. In this case the chromosomes are always small.

Introduction

Parthenogenesis has been long suspected to occur in some tardigrade species. Males are particularly rare in tardigrades that live in moss, and the male to female ratio often varies significantly throughout the year even in species that are found in freshwater (summary: MARCUS, 1929a). Since attempts to cultivate tardigrades failed for a long time, the question whether they exhibit parthenogenesis remained unanswered. BAUMANN (1961) then succeeded in cultivating *Hypsibius convergens*. While he was able to rule out parthenogenesis for this species, parthenogenesis was found to exist in *Hypsibius dujardini*, which was successfully cultivated at around the same time (AMMERMANN, 1962). This species was shown to undergo diploid parthenogenesis, but some cytological processes remained unclear. This paper describes in more detail the maturation of unfertilized eggs of *Hypsibius dujardini*.

We had planned to compare the maturation and development of these eggs with those of fertilized eggs in bisexual tardigrades. Unfortunately, I was unable to obtain them. We therefore used the results from previous publications for this purpose. Most observations on egg maturation were reported by v. WENCK (1914) in her publication on the oogenesis of *Macrobotus lacustris* (likely identical to *Hypsibius dujardini* according to MARCUS, 1929a).

Some information on the meiotic divisions of *Hypsibius convergens* was reported by MARCUS (1929b).

In the meantime, parthenogenesis has also been found in other tardigrade species, specifically *Milnesium tardigradum* (BAUMANN, 1964) and *Macrobotus dispar* (AMMERMANN, unpublished). No information has yet been published on the corresponding cytological processes.

Material and Methods

The animals used for the present research all descended from one female taken in May 1961 from a gross culture originating from *Anlagensee* lake in Tübingen¹. Various algae can be used as a suitable food and substrate. However, they must firmly attach to the bottom of the Boveri dish, thereby permitting the animals to crawl over them. Some chlorococcal algae that were isolated from various samples best met this requirement. These algae likely belong to the genus *Chlorella*. A diluted soil extract (according to Hämmerling, modified) was used as culture solution.

The cultures were kept at temperatures between 21° C and 23° C unless otherwise noted. The animals received fresh water every four days. After 14 days, their locomotion had loosened the algae from the bottom of the dish. The tardigrades were transferred from their culture dishes into new Boveri dishes with a lawn of algae that was approximately four weeks old.

Egg deposition is always associated with the mother molting. The eggs are laid in the exuvium, which keeps the eggs of one clutch together. These eggs are always at the same stage of development. The Bouin-Allen, Petrunkevitch and Susa fixatives proved most satisfactory. The clutches were fixed, rinsed with water and then embedded in agar. Subsequently, the agar block was soaked in paraffin in the customary manner and cut into slices (slice thickness 5µm). We successfully used Heidenhain's hematoxylin for staining.

It is difficult to visualize the chromosomes using the Feulgen reaction. They were best visualized in 5µm thick slices after fixation with a solution of 3 parts ethanol to 1 part glacial acetic acid. Unfortunately, the plasmatic structures were poorly preserved with this treatment.

Results

Hypsibius dujardini has a diploid chromosome number of 10, and the chromosomes are likely telokinetic. The number of chromosomes can be identified by examining the mitoses during embryonic development, particularly the metaphase plates (Fig. 1). Related species have similar chromosome numbers; for example, 10 chromosomes were found in *Macrobotus lacustris* (= *Hypsibius dujardini*?) (v. WENCK, 1914) and 12 in *Hypsibius convergens* (MARCUS, 1929b).

The female stops crawling approximately one hour before depositing the eggs. In the living animal, it is easy to see the eggs in the ovary

¹ I'm very grateful to Prof. Dr. E. MARCUS, São Paulo, for identifying the animals.

with the egg nuclei that are about $6\ \mu\text{m}$ in diameter. Sections show that in addition to stainable thin filaments, the egg nucleus contains a nucleolus that is $3\ \mu\text{m}$ in diameter.

Prophase I starts with the enlargement of the egg nucleus. The 10

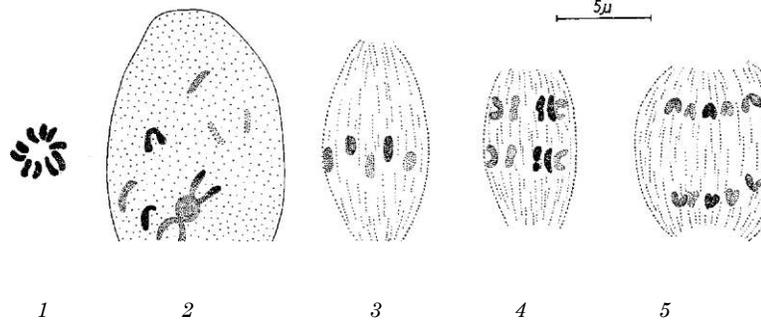


Fig. 1. Metaphase plate of a blastula cell (Petrunkevitch). Fig. 2. Oocyte nucleus, prophase I (Susa). Fig. 3. Metaphase I (Susa). Fig. 4. Anaphase I (Bouin-Allen). Fig. 5. Anaphase I (Stieve)

chromosomes become clearly visible briefly before the nucleolus and nuclear membrane dissolve, which is unusually late. They are still unpaired during this stage that likely corresponds to diakinesis. Two - likely homologous - chromosomes are sometimes seen attached to the nucleolus, which is becoming smaller (Fig. 2). After it is dissolved, the chromosomes pair up. They are too small (around $2\ \mu\text{m}$) for details to be visible.

A small spindle is formed at the beginning of metaphase I. A centriole was not found. Within the spindle, the 5 bivalents arrange to form a ring (Figs. 3, 6). The egg is deposited in this stage. It then immediately rounds up and measures about $40\ \mu\text{m}$ in diameter. Together with the metaphase plate, the spindle subsequently migrates to the egg's surface and orients itself perpendicularly to the egg's surface. At the beginning of anaphase, the spindle becomes wider and shorter. Figures 4 and 5 show two daughter plates that each contain 5 dyads migrating apart. Fig. 5 very clearly shows the dyads with the chromatids that are joined at the kinetochore.

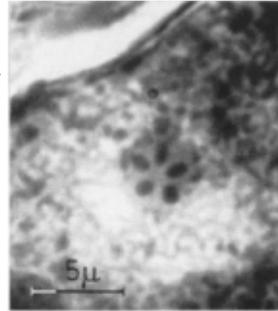


Fig. 6
Metaphase I (Bouin-Allen)

The telophase nucleus located at the margin of the egg forms the first polar body with a small amount of yolk-free plasma. It

is not extruded from the egg and is therefore hard to find in eggs in further stages of development. Only one preparation shows two particularly large polar bodies with 5 chromosomes each (Fig. 7). This suggests that the first polar body occasionally divides again. In the species studied so far, v. WENCK (1914) and MARCUS (1929b) found that the first polar body often divides once more. However, the eggs they examined were fertilized.

It is interesting to observe the further development of the nucleus that remains in the egg. It first forms a nuclear membrane, but it then does not enter

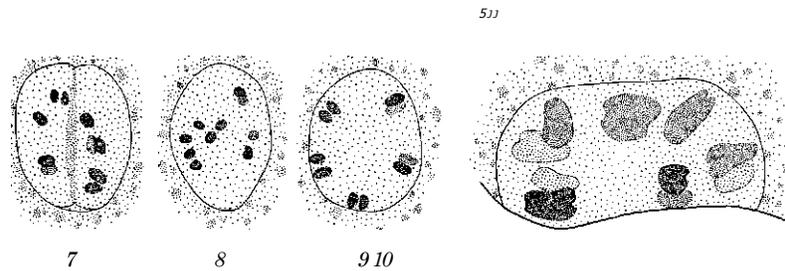


Fig. 7. Two polar bodies (Susa). Figs. 8—10. Start of meiosis II, separation of chromosomes (Bouin-Allen)

interphase. Instead, the 5 dyads separate, forming the 10 daughter chromatids. The diploid chromosome number is thus restored in the nucleus (Figs. 8 – 10). The figures show that the chromosomes may be small (Fig. 9) or large (Fig. 10). This peculiarity of *Hypsibius dujardini*, which can also be observed in the further 5-6 divisions, will be discussed in context below.

The 10 chromosomes initially stay arranged in 5 pairs. After the nuclear membrane dissolves, they finally arrange as a ring and form a metaphase plate (Fig. 11, 12). In the subsequent anaphase, two daughter plates that each consist of 10 chromosomes migrate to the two spindle poles (Fig. 13 – 15). The telophase nucleus located at the margin of the egg is extruded with some plasma as a second polar body. Its formation shows that the division described above was meiosis II. The nucleus that remains in the egg travels to its center. The cleavage divisions then start and proceed as described by v. WENCK (1914) for fertilized eggs.

The chromosome size is always approximately equal for the eggs of one clutch. Different clutches, however, often exhibit remarkable differences in the size of the chromosomes in the karyokinetic figures from meiosis II to the

5th to 7th cleavage divisions. The following figures illustrate this observation: Figures 9 and 10 show two egg nuclei from eggs of different clutches at the end of endomitosis before meiosis II; Figures 11 and 12 show two metaphase plates of meiosis II, and Figures 13 to 15 show the same relationships in anaphase II and telophase II. Fig. 16 shows the differences in size in the two daughter nuclei of meiosis II. In this case, the chromosomes of the egg nucleus likely increased in size during anaphase, while those of the polar body remained unchanged in size. This is suggested by the chromosomes of the egg nucleus often significantly increasing in volume a second time after the polar body is discarded (Fig. 17), which makes them easily visible in the live egg as well.

From endomitosis to the 5th to 7th cleavage division, one can always find eggs with either large or small chromosomes. From the 7th cleavage division onward, only small chromosomes are found in the eggs, as shown in Fig. 1. In fertilized eggs, v. WENCK observed large chromosomes (which she called karyomeres) only in the first cleavage divisions. The presence of large chromosomes as early as meiosis II may therefore be a peculiarity that *Hypsibius dujardini* only exhibits during parthenogenesis.

It is not clear whether the enlargement of the chromosomes has any functional significance at some point during the 6 to 8 divisions or whether the eggs can also develop in the absence of the temporary enlargement of the chromosomes. This question can only be answered by observing the chromosome behavior in individual eggs throughout their entire development, since the chromosomes do not enlarge synchronously in *Hypsibius dujardini*, but large and small chromosomes can be found in each stage of the 6 to 8 divisions. This experiment is not yet possible for methodological reasons, since eggs flattened by pressure do not continue developing, and chromosomes are rarely visible in eggs that are not flattened.

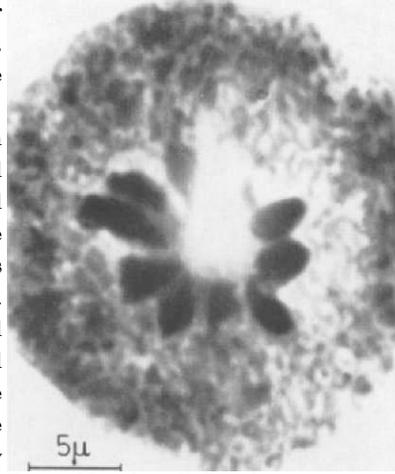


Fig. 11. Metaphase II. Two chromosomes are located outside of the focus range (ethanol : glacial acetic acid 3:1)

It is worth noting that there is no obvious difference in division behavior between large and small chromosomes. Large chromosomes can divide - unlike the somewhat similar karyomeres.

The large chromosomes are not artifacts created during fixation and

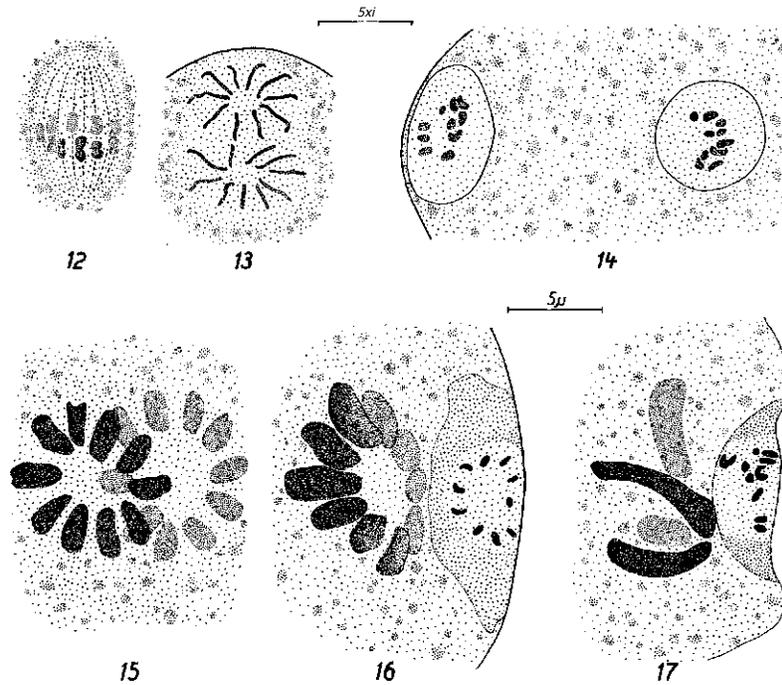


Fig. 12. Metaphase II (Bouin-Allen). Fig. 13. Anaphase II (Susa). Fig. 14. Telophase II (Petrunkevitch). Fig. 15. Anaphase II (Bouin-Allen). Fig. 16. Polar body discarded after anaphase II (Bouin-Allen). Fig. 17. Enlargement of the chromosomes remaining in the egg (only 4 were drawn, 10 present) subsequent to telophase II (Susa)

subsequent treatment, as may be suspected. We often found clutches located next to one another on the same slide exhibiting differently sized chromosomes. Therefore, the prior treatment does not play a role in this respect. The large chromosomes can be found with each of the discussed fixation techniques.

Further experiments provide some information on the molecular composition of the large chromosomes. Applying the Feulgen stain to the sections results in distinctly red staining of the small chromosomes

of meiosis I and other divisions. The large chromosomes, in contrast, are unstained and only visible under the phase contrast microscope. Therefore, they do not primarily consist of DNA. Their DNA is likely finely distributed, resulting in a correspondingly weak staining reaction that is not visible. The methyl green-pyronine stain is not a useful tool for clarifying the molecular composition of the large chromosomes since the egg's plasma stains intensely red. We performed the following experiment to gain at least some insight about the primary composition of the large chromosomes: Numerous eggs were fixed using an ethanol glacial acetic acid solution, cut in slices (slice thickness 5 μm) and stained with Heidenhain's iron hematoxylin. The large and the small chromosomes stained black as they typically do. The remaining plasma remained almost entirely unstained – as usual with this fixative. We then took off the cover slip, removed all traces of the stain from the sections, and treated them with ribonuclease (1 mg/ml at 37°C, 2 hours). Staining again with Heidenhain's stain showed that the small chromosomes accumulated the stain as before, but the large chromosomes only stained very weakly. Therefore, they presumably consist mostly of RNA.



Fig. 18. Anaphase of the second cleavage division (Stieve)

Some anaphase figures from meiosis II and especially those from the first cleavage divisions display a region with stainable particles in the center between the two anaphase plates. This "third plate" can be observed with a variety of fixatives. Fig. 18 shows an anaphase of the second cleavage division exhibiting such a "plate." Its stainable material appears to consist of thin, "granular" filaments that are arranged in the direction of the spindle fibers. The structures are too small for a more detailed analysis. It is striking that any time such a "third plate" is found in an anaphase figure, the corresponding anaphase chromosomes are small. A spindle with large chromosomes was never found together with such a plate of stainable material.

The tardigrade cultures have now been under continuous observation for almost six years. During this time, we did not observe copulation or the presence of males at any time. To ensure that males were not merely overlooked, we isolated freshly hatched animals at regular intervals. They always developed in to egg-laying females. The same is true for cultures that were held at 13°C and 4°C — with some grown in these conditions for months. The animals I cultivated, which belong to one clone, are thus shown to exhibit obligate parthenogenesis.

For comparison purposes, I isolated some animals of this species from an aquarium at the Zoological Institute in March 1963 and from a gross culture originating from *Federsee* Lake in February 1965. To date, we have not been able to find males in those cultures either.

ERLANDER (1895) and HENNEKE (1911) found numerous males and females in two types of tardigrades that may have belonged to the species *Hypsibius dujardini* (see MARCUS, 1929a). However, I was unable to find tardigrades at the locations near Ludwigshafen that were identified by the authors. This may be due to changes (sewage discharge) that these habitats have undergone since that time.

Discussion

All authors who have studied *Hypsibius dujardini* agree that males are sometimes common and sometimes rare but never completely absent. We were therefore surprised to find obligatory parthenogenesis in this species. Cytological examinations have now shown that the animals I cultured appear to have fully “specialized” in this type of reproduction. For the bisexual form, v. WENCK (1914) found that the sperm nucleus remains in the egg after copulation until the egg nucleus has undergone both meiotic divisions, after which karyogamy takes place. In the animals I observed, in contrast, the chromosome number already doubles between the two meiotic divisions. After meiosis II, the egg nucleus is therefore diploid. Given these circumstances, it is difficult to imagine normal karyogamy followed by cleavage taking place in these animals. If this were the case, the penetration of the sperm would have to suppress the separation of the dyads at the beginning of meiosis II. It would therefore be interesting to observe the copulation of males and females of my clone. Unfortunately, I have been unable to find males.

The cytological process of parthenogenesis has already been observed in numerous animals (Summary: SUOMALAINEN, 1950; NARBEL-HOESTETTER, 1964). It was shown that there are various ways of reversing a reduction division - i.e.

returning a haploid egg nucleus to a diploid state. The observations I described for *Hypsibius dujardini* are most similar to those found in some nematodes of the genus *Rhabditis* (BELAR, 1923, 1924; NIGON, 1947, 1949) and those in some oligochaetes, e.g. in *Cognettia* (CHRISTENSEN, 1961). In both groups of animals, the chromatids of each dyad separate at the end of meiosis I. The nucleus then contains the diploid chromosome number, and the egg nucleus is diploid at the beginning of the cleavage division since meiosis II does not take place. The authors justifiably interpret the increase of the chromosome number as a part of the otherwise suppressed meiosis II.

The maturation of *Hypsibius dujardini* eggs differs from this type of parthenogenesis; the division step after the restoration of the diploid chromosome number in interkinesis does not represent the first cleavage division but leads to the formation of a second polar body. Therefore, the separation of chromosomes in interkinesis cannot be interpreted as a “remnant” of meiosis II. However, meiosis II seems like a useless relic in *Hypsibius dujardini*, since it has the character of true mitosis and separates two perfectly identical chromosome sets.

There are still no satisfactory explanations for two observations described above for meiosis II and the first cleavage divisions: The frequent occurrence of large chromosomes and the fact that a third “plate” of stainable material can be observed in some anaphase figures between the two daughter plates consisting of small chromosomes.

These two observations may be interpreted as follows: The chromosomes accumulate stainable material (likely predominantly RNA) in a not precisely defined stage after meiosis I, and they then appear as large chromosomes. In a later division, they discard this material, which remains located wholly or in part in the center between two anaphase plates. Results from other species support this hypothesis. For example, SEILER (1914) researched butterflies and found that a third “plate” made of “elimination chromatin” always remains in the center during anaphase II. BAUER (1933) found that this elimination chromatin is Feulgen-negative in *Ephestia kühniella*. RIS and KLEINFELD (1953) confirmed this result and also found that the elimination material largely consists of ribonucleoproteins. In the mite *Pediculopsis*, COOPER (1939) found that the elimination chromatin is formed not only during meiosis I but also during meiosis II and the first ten cleavage divisions. At the beginning of each division, each chromosome forms a karyomere that dissolves again during division. The chromosomes in anaphase are small, and a Feulgen-negative body, which represents a karyomere remnant, remains in the equatorial plane between them. The similarities between these findings and the observations in *Hypsibius dujardini* are obvious. In *Pediculopsis*, however, these processes take place regularly and synchronously in all eggs, which is not at all the case in *Hypsibius dujardini*. The formation of “large chromosomes” seems to not be strictly tied to a certain stage of development but to take place at any time between meiosis II and the first five cleavage divisions. This complicates an exact analysis of the cytological processes in *Hypsibius dujardini*. Perhaps research on other species will provide more insights.

Summary

1. *Hypsibius dujardini* (*Articulata*, *Tardigrada*) undergoes diploid and – under the given culture conditions – obligatory parthenogenesis. Males were not found.

2. Meiosis I reduces the number of chromosomes. The 5 bivalents separate and are distributed to a small polar body and the egg nucleus. Without interphase, the 5 dyads remaining in the egg nucleus subsequently split into 10 elements, restoring the diploid chromosome number.

3. Meiosis II is equivalent to a normal mitosis. After the relatively large polar body with 10 chromosomes is discarded, the egg nucleus, which also contains the diploid chromosome number, moves to the center of the egg, and the cleavage divisions begin.

4. During meiosis II and the first cleavage divisions, the participating chromosomes are often particularly large. These “large chromosomes” can remain so for several divisions. However, they become small after the 5th cleavage division at the latest. Apparently, their large size is predominantly caused by stored RNA.

5. A layer of stainable material can sometimes be observed between the daughter plates in the anaphases of the first cleavage divisions. In those cases, the chromosomes are always small. This material may be “elimination chromatin” that does not contain DNA and is released when the “large chromosomes” become smaller.

Translation commissioned Jan 2010 by Bob Goldstein, UNC Chapel Hill

Translated by Barbara Bubel, CT, MA

ATA-certified translator, German > English

bubeltranslations.com

Literatur

- AMMERMANN, D.: Parthenogenese bei dem Tardigraden *Hypsibius dujardini* (DOY.). Naturwissenschaften **49**, 115 (1962).
- BAUER, H.: Die wachsenden Oocytenkerne einiger Insekten in ihrem Verhalten zur Nuklealfärbung. Z. Zellforsch. **18**, 254—298 (1933).
- BAUMANN, H.: Der Lebensablauf von *Hypsibius* (H.) *convergens* URBANOWICZ (*Tardigrada*). Zool. Anz. **167**, 362—381 (1961); — Über den Lebensablauf und die Lebensweise von *Milnesium tardigradum* DOYÈRE (*Tardigrada*). Veröff. Überseemus. Bremen A **3**, 161—171 (1964).
- BĚLAŘ, K.: Über den Chromosomenzyklus von parthenogenetischen Erdnematoden. Biol. Zbl. **43**, 513—518 (1923); — Die Cytologie der Merospermie bei freilebenden *Rhabditis*-Arten. Z. Zellenlehre **1**, 1—21 (1924).
- CHRISTENSEN, B.: Studies on cyto-taxonomy and reproduction in the *Enchytraeidae*. Hereditas (Lund) **47**, 387—450 (1961).
- COOPER, K. W.: The nuclear cytology of the grass mite, *Pediculopsis graminum* (REUT.), with special reference to the karyomerokinesis. Chromosoma (Berl.) **1**, 51—103 (1939).
- ERLANDER, R. v.: Zur Morphologie und Embryologie eines Tardigraden. Biol. Zbl. **15**, 772—777 (1895).
- HENNEKE, J.: Beiträge zur Kenntnis der Biologie und Anatomie der Tardigraden (*Macrobiotus macronyx* DUJ.). Z. Zool. **97**, 721—752 (1911).
- MARCUS, E.: *Tardigrada*, in: Bronns Klassen und Ordnungen des Tierreichs, Bd. 5 Abt. IV, 3. Buch. Leipzig 1929a; — Zur Embryologie der Tardigraden. Zool. Jb. (II) **50**, 333—383 (1929b).
- NARBEL-HOFSTETTER, M.: Les altérations de la méiose chez les animaux parthénogénétiques. In: Protoplasmatologia, Bd. VI/F/2, Wien: Springer 1964.
- NGON, V.: La détermination du sexe et la pseudospermie chez un Nématode parth., *Rhabditis monohystera* BÜTSCHLI. Bull. biol. France Belg. **81**, 33—37 (1947); — Les modalités de la reproduction et le déterminisme du sexe chez quelques Nématodes libres. Ann. Sci. nat., 11. Ser., **11**, 1—132 (1949).
- RIS, H., and R. KLEINFELD: Cytochemical studies on the chromatin elimination in *Solenobia* (*Lepidoptera*). Chromosoma (Berl.) **5**, 363—371 (1952).
- SEILER, J.: Das Verhalten der Geschlechtschromosomen bei Lepidopteren nebst einem Beitrag zur Kenntnis der Eireifung, Samenreifung und Befruchtung. Arch. Zellforsch. **13**, 159—269 (1914).
- SUOMALAINEN, E.: Parthenogenesis in Animals. Advanc. Genet. **3**, 193—253 (1950).
- WENCK, W. v.: Entwicklungsgeschichtliche Untersuchungen an Tardigraden (*Macrobiotus lacustris* DUJ.). Zool. Jb. (II) **37**, 365—514 (1914).

Dr. DIETER AMMERMANN
Zoologisches Institut der Universität
74 Tübingen, Hölderlinstraße 12