Review article

Boveri's research at the Zoological Station Naples: Rediscovery of his original microscope slides at the University of Würzburg☆

Ulrich Scheer

Cell and Developmental Biology, Theodor-Boveri-Institute, Biocenter, University of Würzburg, D-97074 Würzburg, Germany

A B S T R A C T

Eric Davidson once wrote about Theodor Boveri: "From his own researches, and perhaps most important, his generalized interpretations, derive the paradigms that underlie modern inquiries into the genomic basis of embryogenesis" (Davidson, 1985). As luck would have it, the "primary data" of Boveri's experimental work, namely the microscope slides prepared by him and his wife Marcella during several stays at the Zoological Station in Naples (1901/02, 1911/12 and 1914), have survived at the University of Würzburg. More than 600 slides exist and despite their age they are in a surprisingly good condition. The slides are labelled and dated in Boveri's handwriting and thus can be assigned to his published experimental work on sea urchin development. The results allowed Boveri to unravel the role of the cell nucleus and its chromosomes in development and inheritance. Here, I present an overview of the slides in the context of Boveri's work along with photographic images of selected specimens taken from the original slides. It is planned to examine the slides in more detail, take high-resolution focal image series of significant specimens and make them online available.

1. The Naples slide collection: an overview

A few years ago I detected hundreds of old microscopic slides in the cellar of our Biocenter which were originally kept in the old Zoological Institute of the University of Würzburg. Among them were slides that had been prepared by Theodor Boveri in the course of his studies on egg maturation, formation of polar bodies, fertilization and early cleavage events. In addition, I found slides containing paraffin sections of dividing *Ascaris* and sea urchin eggs with brilliantly stained centrosomes which most likely had been used by Boveri for his seminal work on centrosomes (Scheer, 2014). Between the stacks of brown cardboard folders of a type still being used today, four elegant book-shape storage boxes with the imprinted inscription *Mikroskopische Präparate* (microscopical slides) stood out (Fig. 1a). The handwritten text on the white labels reads as follows (Fig. 1a, from left to right):

Neapel 1901/02. Versuche an Echiniden-Eiern (experiments on Echinoid-eggs).
Neapel 1911–1912, I.
Neapel 1911–1912, II.
Neapel 1914, II (Box I is missing).

Each box accommodates 100 numbered slots that correspond to a numbered index on the inside of the hinged lid (Fig. 1b). Often two slides are placed back to back in a single slot. The content list is written in Boveri's characteristic, clearly legible handwriting (Fig. 1c). Unfortunately, a number of the listed slides are missing.

The number of slides per box is as follows:

Box Neapel 1901/02: 101 slides.
Box Neapel 1911–1912, I: 175 slides.
Box Neapel 1911–1912, II: 46 slides.
Box Neapel 1914, II: 133 slides.

A further 174 slides dated from 1902 to 1914 are kept in 12 separate folders. Thus altogether 629 Naples slides are still in existence.

The vast majority of the slides are whole mounts of sea urchin embryos stained with borax carmine, but a few paraffin sections are also existent. Two slides taken from the 1901/02 box are shown at higher magnification in Fig. 1d. Depending on the experiment, a slide contains only one or a varying number of sea urchin larvae. In order to provide the necessary distance between slide and coverslip, two hairs serve as spacers. Canada balsam is used as mounting medium. Although it turned yellow over the years, the slides are generally in a surprisingly good condition despite their age. The specimens are well preserved except for their larval skeleton in the form of calcium carbonate spicules which are barely recognizable. It is known that Canada balsam can be acidic which could have caused erosion of the fine skeletal

Fig. 1. Boveri's microscope slides prepared at the Zoological Station in Naples. (a) Four book-shaped storage boxes with handwritten labels (see text). (b) Open box to reveal the slides. Note the inventory list on the inside of the lid. (c) Higher magnification of the numbered and dated inventory list. The heading reads *Neapel, Versuche 1901/01* (Naples, experiments 1901/02). The abbreviations *Disp.-Dreier* and *Disp.-Vierer* stand for "dispermic three" and "dispermic four", i.e., triaster or tetraaster embryos. *Kernlose Fragm.* are nuclear-free egg fragments and *kernhaltige Frag.* are egg fragments containing a nucleus. (d) Higher magnification of two slides from 1902. Note the two hairs each supporting the coverslip in order to prevent flattening of the specimen. Despite yellowing of the mounting medium Canada balsam the optical properties of the slides are generally good. The slide on the left (*15. Jan*) contains three pathological blastulae derived from triaster eggs. The slide on the right (*14. Febr*) contains about 25 triaster *Sphaerechinus* embryos, all arrested at the early gastrula stage (*Gastrulae 16.II. getötet*, i.e. killed on Feb. 16).

structures.

Each slide is dated by Boveri and provided with essential information such as the type of experiment and a brief specification of the content. Some slides bear additional handwritten notes on a second label (Fig. 3a). The meaning of the acronym *NB!* on several slides is not entirely clear (Fig. 3a). Most likely it stands for *Nota Bene* and was used by Boveri to highlight particularly important specimens including those he has drawn for his publications. However, it is quite difficult to identify on a microscope slide those objects displayed in Boveri's publications. This is because Boveri often drew the objects when they were mounted temporarily in glycerol. Slight movements of the coverslip allowed him to roll the object in any direction and thus to integrate all relevant aspects into a single composite drawing. Only after completion of the drawing the object was permanently mounted in Canada balsam.

The slides document Boveri's seminal experiments with sea urchin eggs and embryos, known as the "dispermy" and "merogone" experiments (see below), which provided evidence for the nuclear control of development or, as Boveri put it: "all essential traits of the individual and the species are determined by the chromatin of the egg nucleus and of the sperm nucleus" (Boveri, 1904, p. 113).

Interestingly, the majority of the slides (354 out of 455) are devoted to the merogone experiments (for a comprehensive review see Laubichler and Davidson, 2008). Over many years the Boveris repeated these experiments again and again in order to meet the criticisms by

others and to improve the experimental setup. In his last publication which appeared posthumously three years after his untimely death, Boveri wrote: "On no other experiment have I spent so much time as on the breeding of merogonic hybrids from *Sphaerechinus* eggs" (Boveri, 1918, p. 435). This may explain that the merogone slides outnumber by far the dispermy slides.

2. The dispermy experiments: creating blastomeres with different chromosome sets

Edmund B. Wilson once wrote that Boveri's experiments with double-fertilized eggs "form his crowning achievement, whether in respect to excellence of method or importance of results" (Wilson, 1918, p.74; for reviews see Baltzer, 1962; Sander, 1993; Moritz and Sauer, 1996; Maderspacher, 2008). Boveri's brilliant idea was to produce blastomeres with different chromosome sets and to analyze the effects on embryonic development. In his own words: "We give the cell a nucleus with some parts lacking and follow the effect of this defect" (Boveri, 1902, p. 81).

Sea urchin eggs can be easily fertilized in vitro. When sperm is added in excess, often two sperms enter an egg. These dispermic eggs develop tetrapolar mitotic figures and divide immediately into four rather than two cells. The four spindles compete for the chromosomes with the result that the four daughter cells inherit abnormal and

different chromosome combinations. When eggs are shaken shortly after fertilization, tripolar spindles may also form (mechanical strain obviously interferes with the duplication of the centrosome of one of the two sperms). Such triaster eggs divide simultaneously into three blastomeres and, because of less severe chromosome aberrations, develop better than the tetraasters (a micrograph of a dividing triaster egg is shown in Scheer, 2014). Hence, the triasters allowed Boveri to study developmental defects also in more advanced larval stages.

The dispermy experiments were carried out by the Boveris during the winter of 1901/02 and the results provided compelling evidence for the important role of the nucleus and its chromosomes in heredity and developmental processes. The experimental approach was relatively simple. Sea urchin eggs were collected and fertilized in vitro by an excess of sperm. The fertilized eggs were then transferred into a watch glass and those dividing simultaneously into four blastomeres were isolated and kept in separate culture dishes for further observation. Triasters were produced in the same way except that the eggs had to be shaken shortly after fertilization.

It is interesting to mention that Boveri has studied not only the development of whole embryos derived from dispermic eggs, but also of individual blastomeres. For the separation of the blastomeres he applied the method developed by Herbst (1900). As Boveri wrote, it was Curt Herbst personally who showed him in Naples how to prepare the Ca^{++}-free sea water. Unfortunately, none of the slides have survived. Since they are not indexed on the list of the 1901/02 slide box they must have been stored elsewhere.

In the sixth and last part of his "*Zellen-Studien*" Boveri described the results of 28 different experimental series performed from the end of November 1901 to the end of March 1902 in Naples (Boveri, 1907). Microscope slides contained in the box "*Neapel 1901/02*" can be assigned to 13 out of these 28 dispermy experiments. As an example, the table summarizing Boveri's triaster experiments is reproduced in Fig. 2a. Indicated are the dates of the individual experiments, the species used and the number of objects analyzed. Slides from the experiments No. 1 and 4–11 are existing (a total of 42) and two of them are displayed in Fig. 1d. In contrast, only four slides of the tetraaster experiments have survived.

The triaster embryos were rarely normal. In most cases, their development was blocked at the blastula or early gastrula stage but a certain percentage developed further into all sorts of pathological larvae and even into well-formed plutei. "It is something surprising for the examiner to see how tripartite eggs of identical appearance and cleavage pattern sometimes form a well-shaped pluteus, sometimes an irregular cell aggregate" (Boveri, 1907, p. 79). In fact, the slides contain an impressive gallery of sick, abnormal, asymmetric and decaying objects besides almost normal plutei (Fig. 2b–g).

Cases in which malformations or defects were limited to one third of the embryo proved to be of particular importance. Boveri reasoned that such sectorial defects would arise when only one of the three primary blastomeres carries an abnormal chromosome complement. The faulty chromosome assembly would then be passed on from the founder blastomere to all clonal descendants. Thus, analysis of the phenotypic defect of a larva provided a tool for the analysis of its chromosome constitution. Boveri has termed this approach "*Embryonalanalyse des Zellkerns*" (embryonic analysis of the cell nucleus).

Two examples of sectorial defects are shown in the bottom part of Fig. 2. Cells derived from about one third of the epithelium of a triaster blastula are apparently invading the blastocoel (Fig. 2f). The blastula is arrested at this stage and unable to develop further, as control embryos had already reached the pluteus stage. A comparable situation is shown in the accompanying drawing by Boveri. He writes: "An area which undoubtedly corresponds to a primary blastomere has become pathological and, for the most part, has already entered the inside" (Boveri, 1907, p. 134). Notably, Boveri realized that many of the inner cells appeared to degenerate by a well-defined sequence of nuclear alterations such as chromatin condensation and marginalization into crescent

or ringlike structures (on closer inspection some examples may be identified in Fig. 2f). Nowadays such morphological changes of the nucleus are considered as hallmarks of apoptotic cells.

Another interesting and informative example is shown in Fig. 2g. Here, loss of cell cohesion in about one third of the epithelial cells has caused the local rupture of the otherwise still intact blastula ball, as schematically depicted in the accompanying sketch by Boveri. From this he concluded that the capability to form an epithelial cell layer has been lost in one of the three primary blastomeres. In contrast, when all epithelial cells lost their adhesive properties, then the whole embryo disintegrated into a loose aggregate of cells which, however, retained a normal appearance and continued to divide (Fig. 2d, arrows). The observation that cells may lose their adhesive properties without showing the slightest signs of disease led Boveri to conclude: "Here it has to be assumed that there are certain chromosomes which are necessary for the adhesion of the cells to each other, but otherwise, at least initially, are not of vital importance for the life of the individual cells" (Boveri, 1907, p. 243). No doubt, Boveri was absolutely right. Just substitute the term chromosome for the term gene and the sentence reads: Specific genes are required for cell adhesion- a truly modern view! Even more, in a later article he remarked: "One cannot fail to draw the conclusion that the normal nucleus determines the production of the substance that is required to maintain the cohesion of the blastula cells. If the chromosomes that specify this substance are missing, the cells lose their cohesiveness" (Boveri, 1914, p. 32; English translation by H. Harris, 2007). What a remarkable vision of the process we now call gene expression!

In this context it should be mentioned that Boveri was well aware of the striking similarity between the behavior of chromosomes and Mendel's hereditary factors. Already in 1902 he wrote: "It will have to be concluded that the role of the chromosomes in ontogenesis corresponds rather exactly to the views which have found a brief though not very fitting expression in the designation of these structures as carriers of heredity" (Boveri, 1902, p. 83).

Taken together, the detailed analysis of the dispermic eggs allowed Boveri to conclude: "Thus what remains is that not a certain number, but a certain combination of chromosomes is required for normal development, and this cannot but mean that the individual chromosomes must possess different qualities" (Boveri, 1902, p. 75; English translation by Sander, 1993). The Naples experiments not only laid the foundation for the chromosome theory of development but also let Boveri postulate that tumours might arise by an abnormal chromosome constitution (Boveri, 1914, Harris translation, 2007; for reviews see Manchester, 1995; Balmain, 2001).

3. The hybrid merogone experiments: defining the role of the nucleus in development and differentiation

Already in 1889, during his second stay in Naples, the then young Boveri (at that time he was *Privatdozent* at the Zoological Institute in Munich, headed by Richard Hertwig) performed an experiment which "will always have its significance as a first attempt to define the role of the nucleus and the cytoplasm, and as a brilliantly designed experiment for the solution of a great biological problem" (Baltzer, 1962; Rudnick translation, 1967). During his previous stay in Naples, Boveri followed up the finding of the two Hertwig brothers that shaking brought about the fragmentation of sea urchin eggs into nucleate and anucleate fragments and that after fertilization these fragments began to cleave. Boveri was particularly interested in the development of the anucleate fragments (merogones) and observed that in about half of the cases small, but viable haploid plutei emerged. Thus, the parental set of chromosomes was sufficient for larval development. A step further, he reasoned, would be to fertilize the merogones with sperm of a closely related but morphologically distinct species and to analyze the phenotype of the developing hybrid larvae. Or, as put by Boveri: "We have an enucleated egg…. and we are able by means of a process of
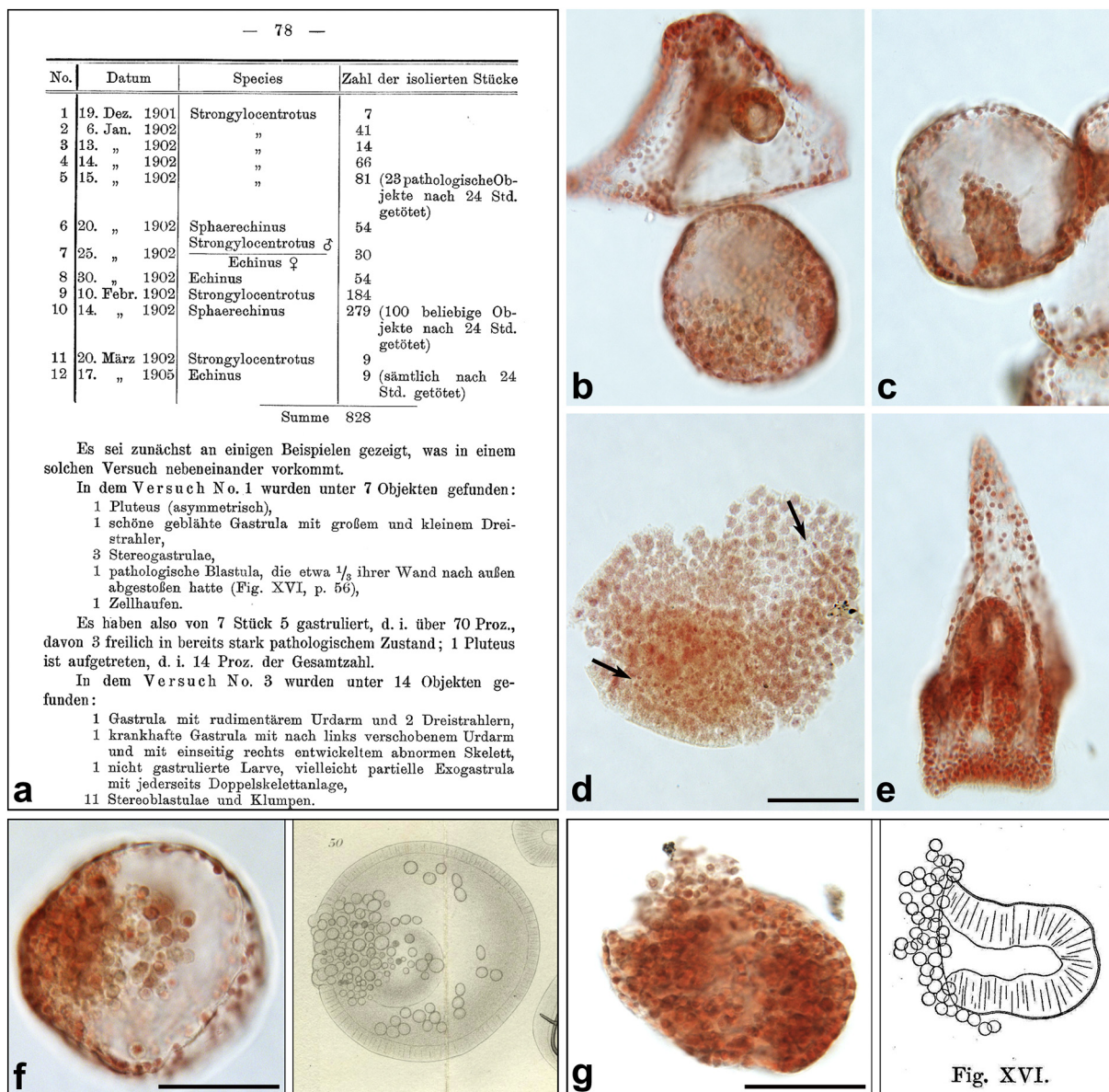
Fig. 2. Development of dispermic embryos. (a) Boveri's table summarizing the developmental fate of a total of 828 analyzed triaster embryos (Boveri, 1907, p. 78). The two slides shown in Fig. 1d belong to experimental groups listed in the table. (b–e) Heterogenous developmental potential of triaster embryos. The majority are arrested at the blastula (b, bottom part) or early gastrula (c) stage, but more advanced embryos at the prism stage (b, upper part) or even pluteus stage (e) are also present. Of particular note are cell aggregates derived from disintegrated embryos indicating the loss of intercellular cohesiveness (d; some cells of the aggregate are still dividing, e.g., at the arrows). (f, g) Sectorial defects of triaster embryos. For details see text. Comparable situations are shown in the micrographs and the accompanying drawings by Boveri (f, right, from Boveri, 1907, his fig. 50 and g, right, from Boveri, 1907, his text fig. XVI). Bars indicate 50 μm (b-e are magnified to the same scale).

fertilization to introduce another nucleus into the egg…It is even possible to bastardize the egg fragments (obtained by shaking) of one species with the sperm of another species, and to rear them far enough to determine whether the developing organism shows the qualities of both species or only of the one species"(Boveri, 1889, p. 76; English translation by Morgan, 1893).

Boveri published his results under the pregnant title "*Ein geschlechtlich erzeugter Organismus ohne mütterliche Eigenschaften*" (A sexually generated organism without maternal characters; Boveri, 1889). The paper attracted much attention in the scientific world and was soon translated by T.H. Morgan (1893). The young Boveri must have been thrilled with the apparently clear outcome of his experiments and one can feel a measure of pride and satisfaction in the concluding statement of his paper "And herewith is demonstrated the principle that the nucleus alone is the bearer of hereditary qualities" (Boveri, 1889, p. 80).

In view of the importance of Boveri's results it is not surprising that several researchers tried to repeat the experiments. However, they came to different results and they questioned Boveri's findings and general conclusions (for details see Baltzer, 1962; Moritz and Sauer, 1996; Laubichler and Davidson, 2008; Maderspacher, 2008). Boveri had to accept the criticism all the more as he himself saw problems with his experimental approach. In particular he had failed to obtain offspring from isolated enucleated egg fragments and therefore had to rely on mass cultures of shaken eggs. In this material he had found small-nucleated dwarf larvae of the paternal type which he considered as descendants of true merogones, i.e. embryos containing only the sperm chromosomes. However, a direct proof was lacking.

Boveri planned to repeat the experiments in order to clarify the controversial points. But due to a number of reasons (illness, appointment to Würzburg in 1893 as director of the Zoological Institute, new administrative and teaching obligations) it was not until winter 1901/ 02 that he took up the merogone experiments again. During this season

Fig. 3. Mass culture of shaken eggs after homogametic fertilization (*Echinus* x *Echinus*). (a) The slide, taken from the 1901/02 storage box (position number 82, see Fig. 1c), is dated March 25–28 and contains *Echinus Fragmente in Massencultur*. Shaking of eggs yielded a mixture of normal eggs and egg fragments with or without nuclei. Hence, after fertilization the mass culture comprised normal larvae and dwarf larvae with either diploid or haploid nuclei. In the example shown, Boveri has isolated several dwarf larvae (*Fragmente*) from the mass culture and prepared whole mounts for microscopic inspection. Written on the right side of the slide is *NB! großkern. u. kleinkern. gleicher Größe neben einander*. (Nota Bene! Equally sized larvae with large and small nuclei side by side). (b) Two juxtaposed dwarf larvae. The nuclei of the larva on the left are significantly smaller and more abundant as compared to the larva on the right. Bar indicates 50 μm.

he carried out not only the dispermy experiments described above but also performed extensive experimental studies on the relations between chromosome number, nuclear size and cell size. Boveri confirmed his earlier observation that haploid nuclei are generally smaller than diploid nuclei. He found two clearly different nuclear size classes in the larvae derived from isolated nucleated or non-nucleated egg fragments after homogametic fertilization (*Echinus* x *Echinus*; a number of slides exists). The same result was obtained when mass-fertilized cultures of shaken *Echinus* eggs were analyzed: the dwarf larvae derived from egg fragments contained nuclei either of the small or the large size class (Fig. 3b; see legend for further details).

By measuring a large number of haploid, diploid and tetraploid nuclei (present in so-called monaster larvae), Boveri established a quantitative relationship between genome size and nuclear size and concluded that the surface of a nucleus is directly proportional to the number of chromosomes it contains (Boveri, 1905). Thus, nuclear size turned out to be a valid criterion and "therefore I could not doubt that the small-nucleated larvae of my 1889 experiment had really emerged from anucleate egg fragments" (Boveri, 1918, p. 419).

Nevertheless, the main objection of his critics was not dispelled and Boveri felt obliged to repeat the crucial experiment which had failed in 1889, namely the interspecies fertilization of <u>isolated</u> enucleated *Sphaerechinus* egg fragments with *Echinus* or *Strongylocentrotus* sperm. The extremely demanding and time-consuming experiments were carried out from January to March 1912 in Naples. Through improved methods, Boveri and his wife now succeeded in cross-fertilizing isolated enucleated eggs and following their individual development (about 40
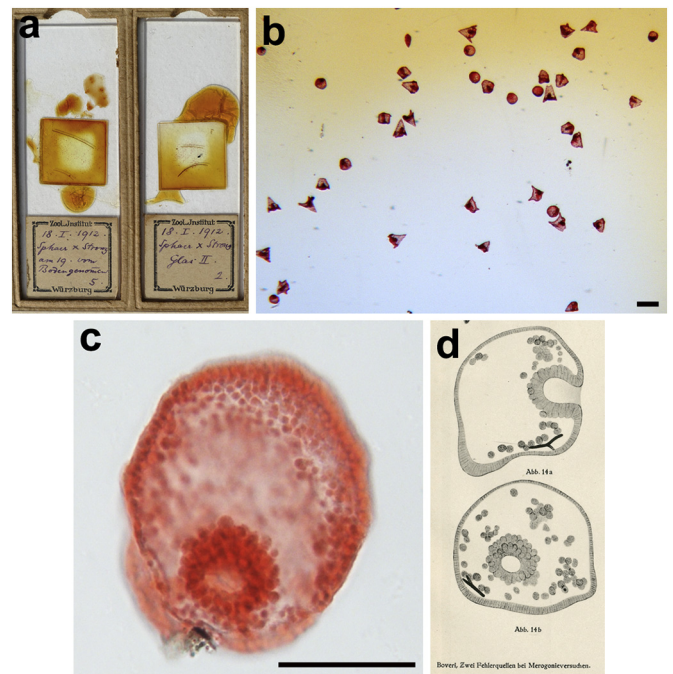

Fig. 4. Development of hybrid merogones in a mass culture. (a) Two slides dated 18. I. 1912. They contain aliquots of mass cultures of shaken *Sphaerechinus* eggs fertilized with *Strongylocentrotus* sperm. The material was taken at various intervals from the bottom of the culture vessel (*vom Boden genommen*, left-hand slide) or from the middle of the *Glas*, i.e., glass vessel (right-hand slide). Note the use of printed slide labels (*Zool. Institut Würzburg*). (b) Survey view of the objects collected from the bottom of the culture vessel (micrograph taken from the left slide shown in a). Early plutei are seen next to stereoblastulae. Bar indicates 200 μm. (c) True merogones, identified by their small nuclei, never developed beyond the early gastrula stage. Bar indicates 50 μm. (d) Comparable situation drawn by Boveri (1918), his fig. 14a + b)

slides of these experiments exist). In essence, the Boveris have analyzed about 200 heterogametically fertilized egg fragments which, by visual inspection under the microscope, appeared to be devoid of nuclei. After 3 to 4 days, the vast majority of the embryos had died or became arrested at the blastula or early gastrula stage. Only 11 embryos developed to plutei larvae. Nuclear size measurements yielded a clear, though unexpected and frustrating result. None of the plutei contained small haploid nuclei. Rather, their nuclei were either of the normal diploid size or represented size intermediates between diploid and haploid nuclei. In contrast, embryos with haploid nuclei never developed beyond the early gastrula stage (Boveri, 1918).

In order to verify the results, Boveri performed a detailed analysis of hybrid larvae obtained in mass cultures of shaken eggs (*Sphaerechinus* x *Strongylocentrotus*). At various time intervals he took samples from the culture vessel and monitored the nuclear size of all larvae. As noted on the slides in Fig. 4a, samples were taken not only from the middle of the glass vessel but also from its bottom (*vom Boden genommen*) where the sick, unhappy and immobile larvae accumulated (Fig. 4b). Initially, all embryos developed up to the blastula stage, independent of their nuclear size. But then further development diverged as a function of nuclear size. The small-nucleated embryos ceased further development. The most advanced hybrid merogone, a 3-day old early gastrula, is presented in Boveri's 1918 paper and reproduced in Fig. 4d. A micrograph of a comparable object (perhaps even the same object?) is presented in Fig. 4c. The embryo is arrested at the first stage of archenteron invagination, apparently unable to enter the process of elongation.

There was only one conclusion to be drawn from these experiments. True hybrid merogones (*Sphaer* x *Strong* or *Sphaer* x *Ech*) do not develop beyond the blastula/early gastrula stage. Hence, the plutei described by Boveri in 1889 could not have been true merogones. Obviously, they were derived from egg fragments still containing some maternal
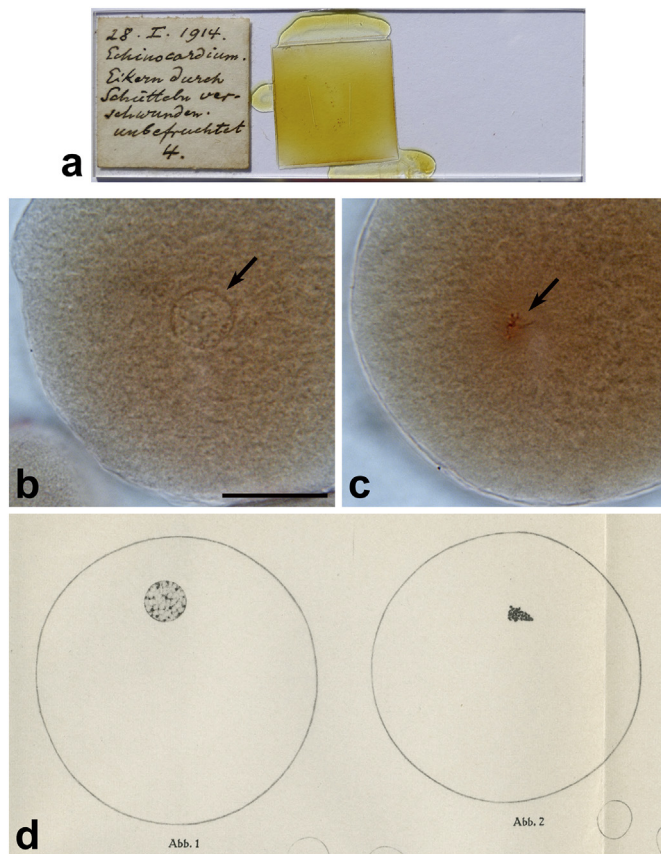
Fig. 5. Microscopic analysis of shaken *Echinocardium* eggs. (a) Prior to fixation, the live material was analyzed according to Boveri's standard procedure and about half of the eggs were classified as nuclear-free. The material was then fixed and processed for microscopy (whole mounts). Micrographs were taken from the imaged slide, dated 28. I. 1914. (b) Egg with a normal pronucleus (arrow). (c) Egg containing a tiny chromosome aggregate (arrow) instead of a nucleus (b and c are magnified to the same scale, the bar in b indicates 20 μm). (d) Comparable situation drawn by Boveri (1918), his figures 1 and 2)

Fig. 6. Multiple partial nuclei in untreated *Echinus* eggs. (a) Slide dated 2. III. 1914. The micrographs were taken from this slide. (b-e) Focal series through a single egg. Arrows denote 4 small partial nuclei located in different regions of the egg. Bar indicates 20 μm. (f) Comparable situation drawn by Boveri (1918), his figures 3–5)

chromatin. "It could hardly be doubted that the fragments isolated as nuclear-free still contained a nucleus. This result – that despite careful work and control by two persons – the nuclei can be overlooked, was rather a knockout blow; it had to be our next task to get to the bottom of this source of error" (Boveri, 1918, p. 422).

At the beginning of 1914, 25 years after his initial publication, Boveri set out to clarify the nature of the "enucleated" eggs by a detailed microscopic analysis of stained whole mount preparations. The findings must have been a shock for the Boveris (see chapter III "The disappearance of the egg nucleus due to shaking" in Boveri, 1918). On January 28, 1914 they prepared whole mounts of *Echinocardium* eggs that were shaken as usual in order to produce fragments with and without nuclei. The shaken eggs were first checked by two persons under the microscope and about half of the eggs were identified as nuclear-free. After inspection the material was fixed by addition of formol, stained with borax carmine and processed for microscopy (Fig. 5a; 6 slides numbered 2–7 are existent).

When the slides are examined in the microscope, eggs with normal nuclei are easily recognized (Fig. 5b, the diameter of the prominent female pronucleus is about 12 μm). In contrast, pronuclei are apparently absent in other eggs. However, on closer inspection and by focusing through the entire egg, not infrequently small chromosome aggregates can be recognized (Fig. 5c, arrow). The same situation is shown in Boveri's original drawing reproduced as Fig. 5d below the micrographs. He described the nuclear remnants as tiny aggregates of stainable substance and (correctly) considered them as densely packed chromatin in a "chromidial state" that has formed after breakdown of
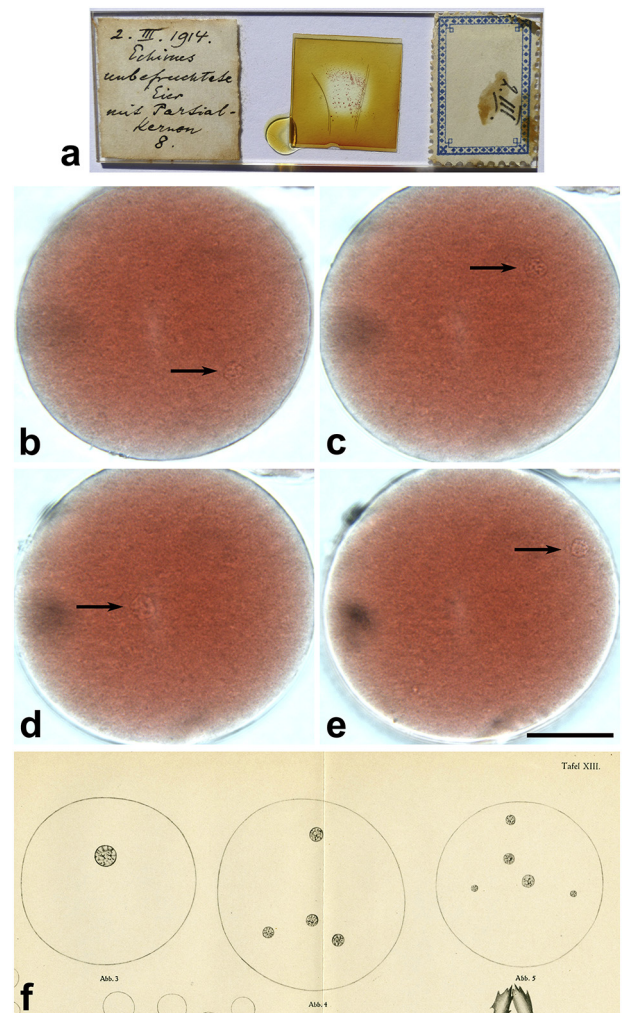
the nuclear membrane and loss of the nuclear sap (Boveri, 1918). He further emphasized that due to their small size such chromosome aggregates could simply escape detection in vivo. Boveri had to conclude that shaking of eggs may lead to the disappearance of their nuclei but not necessarily to the absence of maternal chromatin. Or, in his own words: "*Scheinbare Kernlosigkeit eines Eifragments muß nicht wirkliche Kernlosigkeit bedeuten*".

A second potential source of error came to light when Boveri realized that sea urchin eggs may contain multiple small nuclei rather than a single pronucleus (see chapter IV "The occurrence of partial nuclei" in Boveri, 1918). He observed these so-called "*Partialkerne*" (partial nuclei) in normal (unshaken) *Echinus* eggs (one of the 10 existing slides is depicted in Fig. 6a). Different focal planes of a single egg are shown in order to reveal the presence of 4 partial nuclei (Fig. 6b–e, arrows). Interestingly, the multiple nuclei are not clustered but appear to be randomly distributed within the egg cytoplasm. Boveri's original drawing is reproduced as Fig. 5f. Some of the partial nuclei were so small that Boveri considered them to harbor only one chromosome. Evidently such tiny nuclei could have been easily missed during the selection of "anucleate" fragments. It is remarkable that in addition to describing the partial nuclei, Boveri also discussed their possible mode of origin (which is essentially correct). He was well aware that the formation of pronuclei as well as of the early blastomere nuclei proceeds in two steps. Individual chromosomes first become surrounded by

Fig. 7. One of the last hybrid merogone experiments performed by Boveri in March 1914. Already a few weeks earlier he became aware of the problems with the supposedly enucleated eggs (see Figs. 5 and 6). This probably prompted him to add the words "*die es nicht gibt*" on one of the control slides (encircled in red). The whole inscription reads *Controlle zu kernlosen Fragmenten, die es nicht gibt* (Control for enucleate fragments which do not exist).

a nuclear envelope, thus forming so-called karyomeres (see Fig. 5a and a' in Scheer, 2014). Then the karyomeres coalesce and fuse into a single nucleus. Boveri suggested that when chromosomes are too far apart from each other, not all karyomeres fuse but may remain as individual entities.

The results were published in 1918, three years after Boveri's untimely death, along with a short introductory remark by Marcella Boveri stating that her husband wanted to correct an old mistake he had introduced into science (Boveri, 1918). A remarkable document of frustration is found on a microscopic slide dated March 2, 1914. The slide is part of a whole series (altogether 9) documenting one of Boveri's last experiments in Naples with hybrid merogones. Half of the slides contain whole mounts of hybrid embryos produced from "enucleated" *Echinus* eggs by fertilization with *Strongylocentrotus* sperm. The other half represent controls as indicated in Boveri's handwriting (Fig. 7). At that time Boveri had to realize that nuclear material could still be present in the shaken eggs. Apparently in a mood of resignation he has added on one of the slides the remark "*die es nicht gibt*" (encircled in red). Thus, the complete handwritten note reads *Controlle zu kernlosen Fragmenten, die es nicht gibt* (Control for enucleate fragments which do not exist).

4. Concluding remarks

The microscope slides capture and preserve the information on which Boveri has built a coherent conception of the chromosomes as the carriers of genetic determinants and as the control entities directing the processes of differentiation and development. In particular the experiments with double-fertilized sea urchin eggs represent a masterpiece of meticulous observation combined with logical reasoning and deduction. According to Boveri's friend E.B. Wilson "these works exhibit at their best Boveri's remarkable gift as an observer, experimenter and master of exposition. One who, like the writer, had puzzled in vain over the riddle presented by the double-fertilized eggs of sea-urchins could not read Boveri's complete and beautiful solution without a thrill; and it may be doubted whether a finer example of experimental, analytical and constructive work… can be found in the literature of modern

biology" (Wilson, 1918, p. 75). The results allowed Boveri to propose a mechanism for embryonic differentiation based on differential activation or suppression of certain nuclear qualities. "When the primitive differences of the cytoplasm, as expressed in the existence of layers, are transferred to the cleaved egg without any change in the relationship of the layers, they affect the originally equal nuclei unequally by unfolding (activating) or suppressing certain nuclear qualities …The inequalities of the nuclei, in some cases perhaps of temporary nature only, lend different potencies to the cytoplasm….Thus new cytoplasmic conditions are created which again release in certain nuclei the activation or suppression of certain qualities thus imprinting on these cells in turn a specific character and so on, and so on." (Boveri, 1902, p. 85, English translation by Glueksohn-Waelsch, 1974). Boveri's visionary ideas anticipated the contemporary concept of a genomic program for development based on the dynamic interactions among genes and cytoplasmic gene regulatory factors (for review see Peter and Davidson, 2015).

The microscopic specimens also shed light on the character of Boveri as scientist. Although he knew from the dispermy experiments that his conclusions drawn from the earlier merogone experiments were essentially correct, he nevertheless repeated them again and again in order to meet the criticism raised by several authors (and probably also to resolve his own doubts). Boveri was determined to achieve the goal already set as a young scientist, namely creating hybrid merogones from isolated nuclear-free egg fragments. When, a quarter of a century later, he finally succeeded he had to realize the limitations of the experimental approach. It speaks for Boveri's academic honesty and ethical responsibility that he published not only possible sources of error of the merogone experiments but explicitly expressed his wish that "my earlier statements about merogonic hybrids between *Sphaer* and *Ech* have to disappear from the discussion" (Boveri, 1918).

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