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REVIEW

Professor Giuseppe Reverberi and the ascidian school in Palermo

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Giuseppe Reverberi was born near Perugia on 1901. After being ordained as a priest, he graduated in Theology in 1924 from The Pontificio Ateneo Lateranense. In the same year, he went to the University of Rome, where he took another degree in Natural Science in the lab directed by Federico Raffaele, working on amphibian and chick embryos, under the guidance of professor Pasquale Pasquini. After the degree, he became an assistant at the Zoological Institute where started his studies in the field of experimental embryology, carrying out researches on the chick embryo-eye, on the centrifugation of the amphibian egg and on the potentialities of the amphibian tail bud: these researches were then continued in Palermo by some of his collaborators.

In 1930 he qualified to teach General Embryology and became Professor of Experimental Psychology and Biology at the Ateneo Lateranense. In 1939 he was appointed at the University of Perugia as director of the Zoological institute. In the meantime, he worked at the Zoological Station of Naples, a great attraction pole of international level for marine biology and a breeding ground of scientists. Here he became Director of the Biological Center of the National Council for Research and started to study the first stages of development of the ascidian embryos.

In 1948, as a winner of a public competition, he was appointed as professor of Zoology at the University of Palermo, where he was Director of the Zoological institute and Professor of Biology at the Medical School.

In 1957 he founded the magazine Acta Embryologiae et Morphologiae Experimentalis, a Latin title for an international journal published in English, the first in Italy with an international editorial board. It represented a person whose broad knowledge was not limited to biology, but also covered philosophy, literature and theology. His researches, together with those carried out by the many research groups led by his collaborators were addressed essentially to developmental biology of several animals, overall marine invertebrates: annelids, mollusca (*Dentalium*) ctenophora, amphioxus and ascidians. In 1971 he published a fundamental textbook *Experimental embryology of marine and fresh-water invertebrates* (North Holland) which received a lot of quotations.

For the above mentioned reasons, I wish to speak about the monumental scientific activity of professor Reverberi, which lasted more than 50 years, and of his outstanding human personality. Unfortunately, I did not have the honour to be one of his pupils, but I can remember very well two meetings.

The first one was in 1968, at the Zoological Station of Naples, during an Experimental Embryology course for young researchers: his tall figure, full of charisma, his availability to meet young people coming from different European countries for a dinner party. He was there with the teachers of the School, all the most outstanding embryologists of the 20th century: John Runnstrom, Sven Horstadius, Jean Brachet, Tryggve Gustafson, Gerhard Czihak. I was very impressed by the friendly terms between professor Reverberi and all those distinguished embryologists. It was a very important signal of his international reputation in a period (40 years ago) when not all the Italian Universities used to be so well-known abroad.

I remember the meeting of the Italian Embryological Group in 1977, an atypical club born in 1952 from the idea of some Italian scientists on the model of the Institut International d'Embryologie (IIE). The founders were Pasquale Pasquini, Silvio Ranzi, Alberto Monroy, Alberto Stefanelli, Mario Benazzi and, obviously, Reverberi himself, who was a member of the IIE. During this meeting, professor Reverberi gave a lecture on the latest results and the perspectives of the ultrastructure and the cellular biochemistry of developing embryos, in which he compared and discussed the most recent results obtained in the Palermo lab and in many other labs all over the world . I was very impressed by the fervour of his speech about the communication between nucleus and cytoplasm, one of his favourite subjects.

Ten years later, in 1987, I entered the Institute of Zoology in the historical place of via Archirafi 18: this building was still characterized by his strong personality, even if his illness kept him away for many years. In fact, most researchers were working on ascidians and this was the demonstration that a real School had been created there; a place where the

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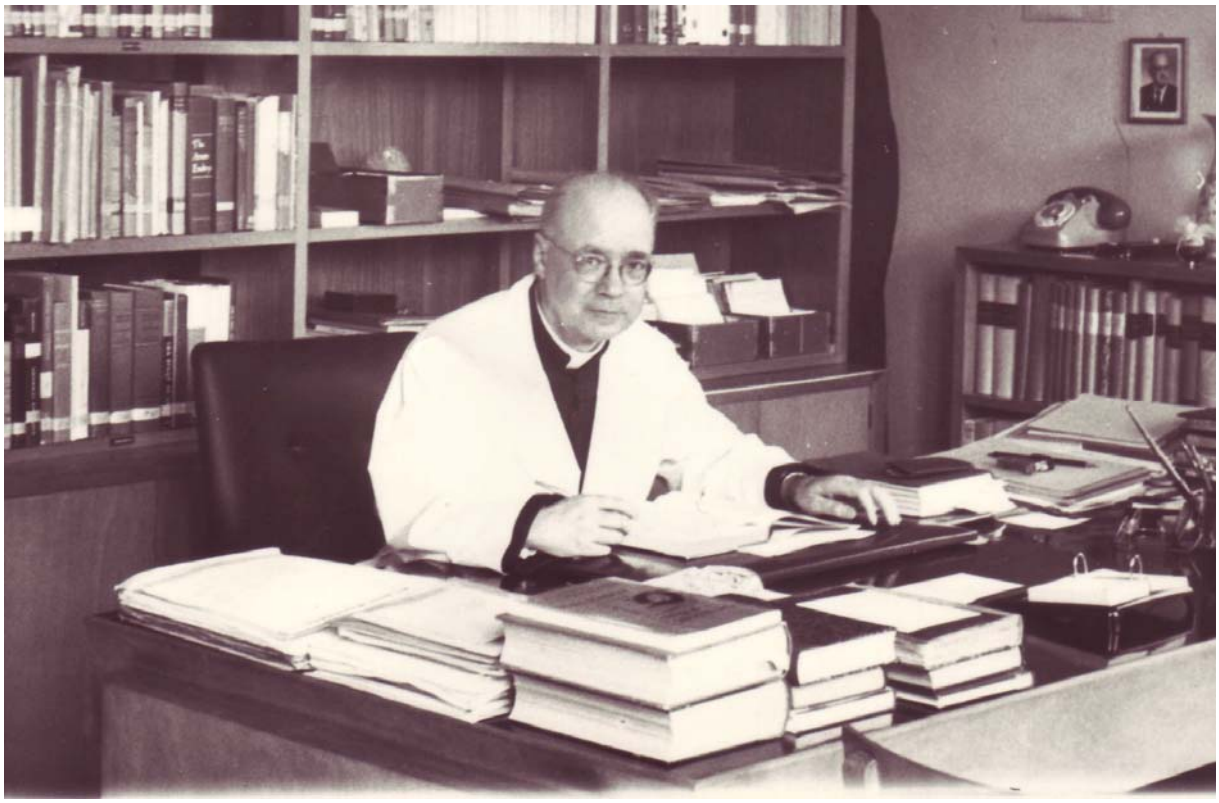

Fig. 1 Prof. Giuseppe Reverberi at work in his room at the Institute of Zoology of Palermo in 1965.

same animal model was studied under manifold point of view. This fact impressed me very much as I was coming from the Zoological Institute of Milan, where the researches used to be more heterogeneous.

It was then inevitable to be overwhelmed by the contagious enthusiasm of his first collaborator and outstanding successor, Giuseppina Ortolani, for the manipulation of the embryos and eggs of the ascidians: one day I told her the technical troubles encountered when highlighting some RNA markers in opaque *Xenopus* embryos, and she said to me:"I worked a lot with *Xenopus* eggs: my advice is to work with *Phallusia*, which has big, transparent and wonderful eggs. Try it and you'll see!" So, I have been working for 20 years with ascidian embryos and I am feeling, even indirectly, an alumna of the famous ascidian school of Palermo!

Reverberi's scientific activity in Palermo mainly involved the experimental embryology field, but the development of ascidian eggs always gained first priority among his interests. In the following sections I will try to define the main research lines concerning the ascidian development.

Development of egg fragments and isolated blastomeres

When Reverberi started to work on the development of ascidians, the current information on such matter was that developed by Conklin, who wrote in the conclusion of his famous paper published in 1905: "The development of ascidians is a mosaic work because there are definitely localized organ-forming substances in the egg; in fact the mosaic is one of organ-forming substances rather than of cleavage cells. The study of ctenophores, nemertines, annelids, mollusks, ascidians and amphibians (the frog) shows that the same is probably true of all these forms and it suggests that the mosaic principle may apply to all animals."

It is interesting to follow the evolution during the time in which Reverberi formed his opinion about the mosaicism based on experimental results.

In one of his first papers, in 1931, Reverberi describes the results obtained from the fertilization of egg fragments obtained by gentle crushing or by a small incision of the chorion. The fragments until 1/22 collected by a needle tip developed "as a whole" and gave normal larvae. His conclusion was "The *Ciona* egg has to be classified among the regulative eggs" (Reverberi, 1931).

1933: blastomeres separated at stages 2,4,8: the animal 4/8 developed blastulae; the vegetal 4/8 developed also gastrulae. "The cleavage is strictly partial (=incomplete), even if some aspects could make suspect of a regulation" (Reverberi, 1933).

1936: "The partial cleavage appears only from the two blastomere stage onwards…; before this stage in fertilized egg the cleavage is of total type" (Reverberi, 1936).

In a frequently quoted paper, even if published only in Italian (Reverberi and Minganti, 1946), we can find some very beautiful drawings, much more explanatory than modern photos. Such drawings showed the results of the selective removal of two

blastomeres each time at 8-cell stage and the histological sections of the larvae obtained from the combination of the animal quartet with the two vegetal anterior blastomeres. This was the first paper unambiguously proving the existence of a "brain evocator" in the two vegetal anterior blastomeres and of a " brain inhibitor" in vegetal posterior blastomeres. These figures became well known as reported in the book of Nori Satoh *"Developmental biology of ascidians"* (1994), a textbook widely diffused in the world of ascidian researchers.

In another paper of the following year (Reverberi and Minganti, 1947), the systematic combinations of blastomeres were described and it was shown that vegetal anterior blastomeres do not "induce" any presumptive epidermis to become brain, but they only "evocate" the neural presumptive ectoderm to become brain.

These works were continued and reconsidered with other collaborators in the following years. They used the knowledge obtained in the meantime by Ortolani about the cell-lineage of the single blastomeres until 64-cell stage and systematically removed the cells committed to give notochord or endoderm from stage 8 to 64. The single cells obtained were then transplanted under an isolated animal quartet. This great bulk of experiments, singularly evaluated, definitely showed that "The formation of the neural system in the ascidians is strictly directed by the same laws which Spemann discovered in the amphibians. Only slight differences are to be noticed between the inductive system in the amphibian and in the ascidians: in the amphibians, the inductor is chorda-mesoderm, in the ascidians it is (part of) the chorda-entoderm". The observation that in the ascidians the inducing power of the chorda-endoderm was more restricted than in the amphibian led to support the idea of the "evocative" power instead of an "inducing" power, enclosed in a bright discussion among the embryologists at that time. These results were reported in another frequently quoted paper "*The causal formation of the brain in the ascidian larva"* (Reverberi *et al.*, 1960).

It is to be noted that this discussion is coming close to a solution only in recent years, after the genome sequencing and the knowledge of the sequences involved in determination of embryonic territories.

Development of sense organs

At the same time of the study on the induction of the nervous system and in the same papers was also studied the development of the principal sense organs: the otholith, the ocellus and the palps. A very elegant result was the cytochemical detection of the DOPA oxidase, the enzyme activating the melanin precursor, only after the induction process at neurula stage. Twenty years later, the problem of the sense organ development was resumed and in two papers was described the ultrastructure of the so-called "third sense organ", probably dedicated to the hydrostatic pressure detection (Reverberi, 1975, 1979). This organ is formed by a few cells bearing bulbous projections projected inside the cavity of the sensory vesicle, it was observed in many species and its function was related to the different ability of the larvae to move vertically along the water column. This topic was also innovative at that time: in fact the third organ was recently studied in several labs, and other hypotheses were developed for its function (e.g., neurosecreting cells or secondary sensorial neurons, Imai and Mainhertzhagen, 2007), but no hypotheses were considered more convincing than the hypothesis originally proposed by Reverberi.

Causal analysis of embryonic development

In a paper of 1939, published in *Commentationes Pontificiae Academiae Scientiarum*, with a latin abstract, Reverberi resumed the experiments of fertilization of egg fragments obtained by centrifugation of the egg. The centrifuged eggs were divided in two parts, one completely hyaline and the other full of granules. Only the latter, after fertilization, can develop even into larval stage, but the hyaline fragment cannot start any cleavage. From these observation, Reverberi inferred that the construction of an organism starting from the zygote is the result of an ordered series of biochemical events. During the development of the ascidian egg the different constituent of the egg become more strictly segregated (plasm segregation) (Reverberi and Pitotti, 1939). The further development of these researches, carried out with collaborators, led to the cytochemical study of the segregation of RNA and DNA. In a review of 1960, published in *Advances in Morphogenesis*, Reverberi states to trust the evidence that differentiating tissues have a higher concentration of RNA (e.g., neural tissue at neurula stage), but he is sceptical about a different concentration of DNA. In fact, at that time, the dispute about the constancy of the DNA content was still going on. Reverberi suggested that "the strong coloration of the nuclei of the nervous cells is not necessarily due to a higher content of DNA, but, more likely, to the fact that the nuclei are more condensed".

Interest was also shown in the mitochondrial respiratory enzymes. Through a very accurate Nadi-reaction and through various cytochemical reactions, it was observed that many enzymes accumulate following mitochondrial segregation in the yellow crescent, in vegetal posterior blastomeres, and in the musculature of the larva (Reverberi, 1957a). The role of the enzymes in ascidian morphogenesis was established by blocking their activity with specific inhibitor. The main results were to ascertain a causal relation between the enzymatic activity and the differentiation of the muscular system, rich in mitochondrial enzymes. In fact the larvae obtained from the treatment with inhibitors showed a normal trunk, and a tail with various anomalies, all referred to a defective differentiation of the muscular fibers (Reverberi, 1957b).

Also related to the previous ones, were the results of the cytochemical reaction for cholinesterase which can be noticed at neurulation not in the neural plate, as previously reported by other researchers, but in the muscular territories.

After the publication of these pioneer results (Durante, 1956), the acetylcholinesterase as a marker of muscular differentiation was used by many authors in many labs (e.g., in USA by R Whittaker, who visited several times the Zoological Institute in Palermo, and in Japan by N Satoh).

Heterospecific hybridizations

A lot of work was dedicated by Reverberi to the heterospecific hybridizations. The results highlighted that, in order to obtain heterospecific fertilization, the eggs have to be naked, without chorion and follicular cells. The latter not only favour the floating of the eggs, but have also a function in species-specific recognizing..

The viable hybrid andromerogons (an enucleated egg of *Ascidia malaca* fertilized with a sperm of *Phallusia mammillata*) have only one set of paternal chromosomes. The morphological characters of the larva are, however, matroclinous, as the cytoplasm was "conditioned" by its nucleus much earlier, perhaps in the ovary. It must be noted that similar results have been observed in other labs on Echinoderms.

Some studies carried out from 1955 to 1960 tried to explain this phenomenon by developing the embryos in sea water additioned with thymidin, adenine etc, in order to identify possible errors in the syntesis of DNA and RNA. But these experiments were too advanced at that time and the transcriptomic and proteomic were still far.

Very advanced were also the experiments on the fusion of the eggs of the same species or of two different species and subsequent fertilization with an heterospecific spermatozoon: giant triploid larvae were obtained and some time these larvae metamorphosed in juveniles (Farinella *et al.*, 1969).

In order to better understand the great interest of Reverberi for these studies it is important to remember that three chapters of his textbook *"Introduzione all'embriologia sperimentale"* (1967), are dedicated to the heterospecific hybridizations. All the problems connected with the suppression of one of the two sets of chromosomes or their maintenance and integration, the "conditioning" of the diploid nuclei from differentiated cells and transplanted in the eggs, were debated in the sixties and had an intellectual appeal that can be grasped in the textbook. It is clearly resumed in the definition "the egg is an equipotential armonic system".

In the first chapter of the above mentioned book, entitled *"Birth and development of the embryology"* a complex and fascinating historical account is outlined with clarity of style and the numerous Latin quotations demonstrate that Reverberi was a very cultured and intellectual person.

Effects of extraneous substances

Through a fusion of the two main research fields, the classical experimental biology and the chemical embryology, Reverberi also tried a new way of manipulating the embryos: instead of using the glass needles, he used some chemical substances. The first of these new research lines on ascidian embryos was carried out with a classical, historical substance: lithium chloride (Farinella Ferruzza, 1955). Treatment of unfertilized eggs produced normal larvae, but a treatment at stage 2 blastomeres gave rise to larvae lacking the trunk and all ectodermal derivatives, like nervous system and palps. The treatment at stage 64 gave apparently normal larvae, but deprived of nervous system and palps. The well known "vegetalizing" action of lithium chloride produced the absence of the neural induction (Reverberi and Farinella Ferruzza, 1961). This research line was further developed and extended by some collaborators who also studied the effects of the environmental pollutants.

Another research line was the treatment of the embryos with chromomycin, actinomycin D or with aminoacids and their analogs, in order to modify the quality of the aminoacid pool leading the embryos to produce abnormal proteins. In all these cases a normal development was obtained when the treatment was carried out before the first cleavage. Treatments in further stages until gastrula produced larvae with various degree of abnormalities (Bramachary and Reverberi, 1964). The results obtained by Reverberi, together with the other results obtained in those years on amphibians, sea urchins and chickens were fundamental to establish that the transcription of embryonic RNA starts at the blastula-gastrula stage and that the ascidians, even if traditionally considered examples of the mosaic development, "have many regulative aspects" as Reverberi already wrote in 1932.

The ascidian embryo ultrastructure

In the fifties, electron microscopy was still in a pioneer phase when the first papers on the ultrastructure of the oocytes, of the unfertilized egg and of follicular and test cells were published. Vincenzo Mancuso and his collaborators worked actively in this research line.

Bearing in mind the lines of the previous researches, we shouldn't be surprised that the electron microscopy was utilised to study the fine structure of the egg and of its constituents separated by centrifugation, in order to study directly what was inferred by cytological and cytochemical methods (Reverberi and Mancuso, 1960; Mancuso, 1963). Once established the unequal distribution of principal constituents of the cytoplasm, their segregation was observed during the cleavage in lineage of blastomeres defined by their morphogenetic commitment. In particular, the cell-lineage of the muscular cells, already outlined in the experiments of Ortolani, was confirmed by the electron microscopy, from the richness in mitochondria of the committed blastomeres .

I would like to mention a series papers on some "peroxidase cells", so identified by Ries through cytochemical reactions. These cells are present in two rows, between the ventral surface of the pharynx and the epidermis, in the mature larvae only in the species carrying a heavy tunic, such as *A. malaca, P. mammillata, Ascidiella aspersa.* They have been considered of mesodermal origin, but Materazzi and Ortolani (1969) demonstrated that

they develop from the anterior vegetal blastomeres, an endodermal territory. These cells cross the ectoderm and join the tunic, where are probably active in the synthesis of mucopolissaccharids of the tunic. Reverberi reported the electron microscopy observation of these cells, just sorted from the endoderm and rich in Golgi apparatus and vesicles (Reverberi, 1971). One of my first papers on the ascidians, carried out with the enthusiastic advice of Ortolani, was about the cytoskeletal modifications of these "button cells" studied by immunofluorescence and confocal microscope while they penetrate the ectoderm (Sotgia *et al.*, 1993).

Immunobiology

Ascidian immunobiology has recently become very important in the Department of Animal Biology in Palermo but, surprisingly there are no studies on this matter in the papers of Reverberi. Precisely, this research line began with an idea of Nicolò Parrinello who decided to carry out the study on serum proteins through immunological techniques in order to solve some taxonomy problems. Professor Reverberi understood immediately the potentiality of this line and encouraged him to continue. In fact, one of the duties of a real Master is also to support self-esteem and to encourage autonomy in young researchers!

Naturally, speaking of Reverberi involves mentioning his collaborators and his School as well. In fact, all his students soundly developed their analytical skills thanks to their Master, following and sometimes being ahead of the new research orientations suggested by the technical progress in biological research.

In 2003, during the first International Urochordate Meeting, Nori Satoh, in the opening lecture *"Let's move on ascidian biology with new ideas*", made an historical review on the ascidian researches and recalled the major scientists: Chabry (1887), Conklin (1905), Reverberi (1931) and his collaborators Ortolani and Minganti.

The meaning of this historical parade was clearly explained during the lecture: the School of Palermo was leader in the experimental embryology for more than 50 years, mainly studying the ascidian development: its heraldic animal.

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