Chapter 1

# Introduction

The history of fertilization is as fascinating as the subject itself, stretching from Greek philosophers through medieval times to the present day, raising many passionate controversies between scientists and philosophers that are often tainted with religious beliefs. Advances were due in part to the invention of scientific instruments, such as the light microscope of Leeuwenhoek, but the main thrust has been the intuitive curiosity of some outstanding scientists. Although diagnostic tools are different today, the gametes are not. We can rest assured that, when observing gametes, what passes through our imagination captured that of our predecessors and to them we should always give due credit.

Hippocrates (460–370 BC) argued that both male and female 'semen' existed and that these mixed in the uterus to form an embryo. Aristotle (384–322 BC) considered animals to be divided into two groups: the bloodless kind, such as insects which generated spontaneously, and all the others that had to mate in order to reproduce. Aristotle favoured a male-centred view where, although the female provided the matter through her menstrual blood, the male semen gave form to the matter. The ideas of Aristotle and Hippocrates dominated thought in the Western world for over 1,500 years until the English physician William Harvey (1578–1657) published his landmark book *De Generatione Animalium* in 1651. The frontispiece of the book depicts Zeus holding two halves of an egg inscribed with the words *ex ovo omnia,* with plants, insects, fish, amphibians, reptiles, birds and mammals emerging from the shell. The concept 'Everything comes from the egg' gained ground over the next 25 years and was supported by the observations of Francesco Redi in 1668 and J. Swammerdam in 1669, both of whom worked on insects. Reinier de Graaf in 1672, provided a detailed account of the human female reproductive tract and, from studies mainly on the rabbit, suggested that ovarian follicles were in fact eggs that were found in the fallopian tubes after copulation. It was not until the early nineteenth century (1827) that the Estonian Karl Ernst von Baer actually observed the mammalian oocyte under the microscope and illustrated the oocyte lying in the Graafian follicle of the ovary of a sow.

In 1677, a Dutch draper, Antonie van Leeuwenhoek, who had begun making simple single-lens microscopes, observed tiny animalcules in his own semen that later were given the name 'spermatozoa' (which translates as 'semen animals'). Leeuwenhoek did not immediately grasp the importance of the discovery of spermatozoa and thought they were another example of animalcules which were found in other biological material, including pus cells. He did however, in 1699, fight the notion that the spermatozoon contained a preformed human, the homunculus, and concluded that there were two sorts of animalcules, one female and one male.

Lazzaro Spallanzani, an Italian priest, published his 1785 classical work *Expériences pour server a l'histoire de la génération des Animaux et des Plantes* in Geneva and was the first person to successfully carry out artificial insemination. He also investigated the effect of temperature and certain chemicals on the fertilizing power of spermatozoa from amphibians. For example, he showed that toad sperm lost its fecundity after six hours at 70°F, but remained fertile for up to 25 hours if it was kept in an icebox at 40°F (the forerunner of today's cryobiology). As a consequence of this latter work, he tried to trigger development with a variety of chemical agents; in fact he introduced the first experiments on artificial parthenogenesis. Although he is often quoted as the scientist who promoted the idea of the activating capacity of spermatozoa, he actually held the opposing idea. In experiments, he showed that if seminal fluid was filtered through filter paper, the filtrate had no fertilizing power, whereas the residue would fertilize. He concluded wrongly that filtration removes the fertilizing power of seminal fluid and sustained that the fertilizing power must remain on

the filter paper. Spallanzani missed the fundamental conclusion that the spermatozoa themselves were the fertilizing agents.

In the first half of the nineteenth century, two main fertilization theories circulated. In 1824, Prevost and Dumas proposed that the spermatozoon actually penetrated into the egg, while Bischoff in 1841 supported the idea that the spermatozoon acted by contact only. George Newport in 1853 showed in amphibians that it was the spermatozoon *not* the seminal fluid that 'was the sole agent of impregnation' (p. 231). After long years of study in which he was adamant that the spermatozoa penetrated the layers of the oocyte, he finally detected spermatozoa in the oocyte's 'yelk'; the oocyte cytoplasm. However, Newport did not directly observe penetration of the spermatozoon and believed that many spermatozoa were required for fertilization, stating 'Fecundation is not the result of a single isolated spermatozoon' and 'a plurality of spermatozoa is necessary for the full impregnation of the egg and the production of a robust and healthy embryo' (1853, p. 245). Newport's work was fundamental in showing that gamete function was dependent on time after ovulation or ejaculation and was also temperature dependent. He also describes the first activation event in amphibian oocytes – that is, the formation of a space or 'chamber' between the vitelline envelope and 'yelk' within 90 minutes of activation and showed that this was in fact located at the animal pole where sperm penetration was preferential. Around the same period, others also maintained they had observed penetration of the ovum by the spermatozoon, such as Barry in 1840, Meissner in 1855 and Keber in 1854, who placed special emphasis on the micropyle seen in some animals as an adaptation for the entrance of a spermatozoon.

The first direct evidence for sperm penetration was not made until 1879 by the Swiss zoologist Hermann Fol using the starfish *Asterias*. Fol observed a thin filament extending from the spermatozoon through the jelly layer of the oocyte to the oocyte's surface. Although Fol dismissed the idea that the filament arose from the spermatozoon itself, he alluded to the fact that the filament pulled the spermatozoon to the oocyte's surface. He also observed that, as the spermatozoon moved through the jelly, a protrusion from the oocyte's surface, the fertilization cone, appeared to rise and meet the oocyte. Oskar Hertwig in 1875, taking advantage of the remarkable clarity of sea urchin oocytes, described one of the fundamental phenomena of fertilization, the sperm nucleus and its aster with the approach of the sperm nucleus to the female nucleus and their apparent fusion. In 1883, Van Beneden, in his classical paper on the parasitic nematode worm *Ascaris*, showed that the pronuclei do not unite but are included in a single amphiaster and that each pronucleus produces two chromosomes. He thus demonstrated for the first time that there are equal numbers of male and female elements in the nuclei of the early embryo.

Theodor Boveri in 1887–1888, again using *Ascaris*, stated 'the egg is devoid of the organ of cell division, the centrosome; capacity for division, hence the initiation of the developmental processes, is restored through the introduction of a centrosome into the egg by the spermatozoon' (see Lillie 1916, p. 48). The paternal control of cell division was thus introduced. In the Stazione Zoologica in Naples in 1888, Boveri, now using the sea urchin, not only promoted his theory on the role of the centrosome in fertilization and early development, but he also discovered the jelly canal that marks the animal pole and showed that 'normal development is dependent on the normal combination of chromosomes and this can only mean that the individual chromosomes must possess different qualities' (see Lillie 1916, p. 48). Later in 1901, Boveri observed a ring of pigment in the oocyte of the sea urchin *Paracentrotus lividus* and related the polarity of the larva and cleavage to this equatorial ring. From this he recognized the importance of the vegetal cytoplasm and the micromeres 'that the area nearest the vegetal pole possesses the greatest potential to bring development to the pluteus stage' (see Ernst 1997, p. 253). These observations were clearly the precursor to the concept of the organizing centre forwarded by his future student Hans Spemann in 1924.

Thus by the end of the nineteenth century the morphological analysis of fertilization was fairly complete. Shortly afterwards scientists attempted to imitate the action of the spermatozoon by chemical and physical agencies. The scientists of the day coined the term 'irritable protoplasm' to describe the ease with which the oocyte surface could be altered. Embryonic surface waves, although previously noted by Fol in 1887, were first described by E. Conklin in 1905 in the ascidian *Cynthia partita*. These, in fact, were the mechanical manifestations of what we now know as

the calcium waves that are generated in all oocytes at fertilization. In 1919, Ernest Everett Just, the first black American scientist, showed that 'before the actual elevation of the fertilization membrane, some cortical change beginning at the point of sperm entry sweeps over the egg, immunizing it to other sperm'. In 1939, in his landmark book, Just suggests this change may be attributable to nerve conduction, 'because among animal cells it is the most highly excitable and the most rapidly conducting' (p. 114).

Frank Lillie in 1916 introduced the quantitative aspect of fertilization, noting that the reaction may exhibit varying degrees of incompleteness. Lillie also states a fundamental rule in fertilization, that is, the spermatozoon will not fertilize until it is fully differentiated. Jacques Loeb, at the beginning of the twentieth century, showed that ion concentration and type were important for fertilization, starting the trend of chemical embryology, and in 1913 he successfully activated sea urchin eggs with butyric acid, resulting in normal cleavage and complete parthenogenetic development.

The undisputed innovator of experimental embryology, Sven Horstadius, actively published in the field for over 50 years from the 1920s to the 1970s. He created the first fate map of early sea urchin development and is known for his blastomere isolation and transplantation experiments. He showed that the entire embryo did not form without cells from the vegetal region. Advances in the detection of nucleic acids led to Jean Brachet showing in 1933 that sea urchin eggs must contain both DNA and RNA, and he came to the conclusion that nucleic acids must take part in the synthesis of proteins. Brachet sustained that the sea urchin oocyte was as an ideal organism for the study of this new area of molecular biology, well before the discovery of the structure of DNA by Watson and Crick in 1953. Finally, with the advances in electron microscopy in the 1950s and 1960s, A. Colwin and L. Colwin from the United States and J. Dan from Japan painstakingly described the various stages of the acrosome reaction in many invertebrates from starfish to polychaetes, pinpointing this reaction as the key to successful sperm–oocyte interaction.

Medical and veterinary interests promoted research in mammalian fertilization in the early twentieth century when the Russian School of Ivanov developed artificial vaginas and insemination techniques to be used in horses, cattle and sheep (surprisingly over a hundred years after the first application of the technique by Spallanzani). Since then, the use of artificial insemination techniques has progressed rapidly until the present day. Now, techniques of in vitro fertilization (IVF), embryo culture, cryopreservation techniques and genetic assessment of gametes and embryos are widely applied throughout the world. A major leap forward in this technology was made in the late 1950s, when the team led by Chris Polge in Cambridge, England, developed techniques to freeze and store animal spermatozoa (some 200 years after the discovery of Spallanzani). This same period of time also saw the development of methods to isolate and manipulate the female gamete. In vitro maturation of mammalian oocytes was first reported by Pincus in 1935, when it was observed that the primary oocyte of the rabbit resumed meiosis spontaneously when liberated from its follicle and placed in a suitable culture medium. It was not, however, until 1968 that Joe Sreenan in Ireland observed in vitro nuclear maturation in bovine oocytes recovered from slaughterhouse cattle. The present day IVF technology derives from the birth in 1978 of Louise Brown in the UK from human embryos produced in vitro (Steptoe and Edwards 1978). While, assisted reproductive technologies (ART) have been developed primarily to alleviate sterility, the possibility of having human gametes in vitro has led to a surge in pure research on human gametes and fertilization.