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Chapter I

Gametogenesis, Embryonic and Post-Embryonic Development of Drosophila Melanogaster, as a Model System for the Assessment of Radiation and Environmental Genotoxicity

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Abstract

Gametogenesis in all animals, seems to be a much more sensitive developmental stage than later stages of their lives. In *Drosophila*

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melanogaster, gametogenesis -especially oogenesis - is shown to be a very sensitive biological phase, in response to exogenous stress factors like radiation - including electromagnetic fields (EMFs) - chemicals, elevated or low temperature, food deprivation, etc. Stress-induced cell death during oogenesis is already studied for a variety of stress factors. The high sensitivity of oogenesis, in combination with the fact that particularly in *Drosophila* it is very well studied, makes this animal a valuable tool for the study of the biological activity of different environmental agents. The developmental stages of oogenesis, embryonic, and post-embryonic development in *Drosophila* exhibit a good timing under certain environmental conditions, and this has led us to the design of experimental protocols based on the assessment of the reproductive capacity, by the number of the first filial generation (F_1) pupae, reflecting the effect of the different exogenous factors on gametogenesis and, in turn, on oviposition. Due to the existence of special checkpoints during oogenesis (most sensitive developmental stages), all the abnormally developing eggs are properly eliminated by stress-induced cell death on the nurse and follicle cells during early and mid-oogenesis leading to the destruction of the whole egg-chamber. Thus, usually, environmental factors do not have an effect on the offspring's genome and morphology but rather on the number of laid eggs (oviposition) and, consequently, on the number of developing post-embryos (larvae and pupae). For this, the number of F_1 pupae, under certain conditions, may reflect biological changes due to exogenous factors. The situation may be more complicated when the exogenous stress factor is microwave radiation. This kind of man-made radiation is found to damage DNA not only in the nurse and follicle cells during oogenesis as by other previously known stress factors, but also in the oocyte from which the progeny organism will be developed after fertilization. DNA damage in the oocyte may result in inherited mutations transferred to the next generations and for this reason the biological changes due to microwave radiation may not be restricted only to changes in the reproductive capacity. In this chapter, *Drosophila* gametogenesis, embryonic, post-embryonic development and life-cycle are briefly described. The chapter then focuses on stress-induced cell death during oogenesis, and discusses a basic experimental protocol regarding the effect of environmental stress factors on reproductive capacity, based on the increased sensitivity of *Drosophila* gametogenesis and the good timing of the embryonic and post-embryonic developmental phases.

Keywords: Drosophila, oogenesis, spermatogenesis, gametogenesis, embryonic development, post-embryonic development, reproductive capacity, biological effects, DNA damage, cell death, apoptosis, radiation, electromagnetic fields, environmental stress.

Introduction

Cellular functions in insect cells are identical as in mammalian cells and moreover, intriguing gene similarities exist between insects and mammals. Besides, insects and particularly *Drosophila*, are more resistant to certain types of environmental stress - such as radiation - and have a much shorter life cycle than mammals. In *Drosophila*, we can observe the effect of an external stress factor on reproduction or on the offspring's genome within a few days, while even in the smallest mammals we have to wait for months. If we add to these advantages the very good timing of the successive developmental stages under certain laboratory conditions and the fact that particularly *Drosophila* is a very well studied organism - probably the best genetically described animal - we can see why *Drosophila* is one of the most frequently used animals in biological experiments.

Drosophila melanogaster (also called fruit fly), belongs to the dipteran class of holometabola endopterygota insects. These are insects with a pair of wings which develop within the body, with a pupal stage in their life cycle and significant morphological changes during metamorphosis from the post-embryonic developmental stages (larval and pupal) to the adult stage.

The length of the wild-type adult insect's body is 2-3 mm and consists - as in all insects - of three distinct basic regions: a) The head with a pair of antennas, mouth parts and two red compound eyes, each one consisting of about 780 repeating hexagonal units called ommatidia. b) The thorax, with three clearly delineated segments (prothorax T1, mesothorax T2, and metathorax T3) each one bearing a pair of legs, a pair of wings on mesothorax, and halteres (remnants of a second pair of wings) related to maintenance of stability during flight by adjusting the wings to destabilizing forces. c) The abdomen, with eight clearly visible parts (A1-A8).

The males have a slightly smaller abdomen and distinct black spots in the front pair of legs. It is a vegetarian insect with suctorial mouth parts. The whole life cycle from egg fertilization to the death of the adult insect usually lasts about a month and occasionally it may extend until two months.

Gametogenesis-Fertilization

Gametogenesis is the process of development of the reproductive cells (gametes) of male and female, i.e. spermatogenesis and oogenesis.

Spermatogenesis starts in the male *Drosophila melanogaster* insects during embryonic development, and by the hatching of the larva from the egg there are already 36-38 spermatogonia in the pair of primary testes (Ashburner and Wright, 1980).

Spermatogonia become surrounded by mesodermal cells to form spermatocysts. Within a cyst each spermatogonium undergoes four mitotic divisions followed by meiosis to produce 64 spermatids. The meiotic division in the first spermatogonia, takes place at the beginning of the pupal stage, about 96-120 h after the hatching of larva from the egg (Ashburner and Wright, 1980).

The spermatids, initially interconnected by cytoplasmic canals, are eventually transformed into mature sperm cells. During this transformation, an acrosome of condensed chromatin forms at the anterior end of each spermatid's nucleus. At the posterior side of each spermatid all the mitochondria in the cytoplasm form a tubular structure around an axial filament ("axoneme") extending from the nucleus to the distal end of the cell (spermatid). The anterior end of the axial filament is bound to the nucleus by a centriole. The axoneme with the surrounding mitochondrial structure become elongated forming the "tail" of the mature sperm cell. The mitochondrial structure gets condensed in several points along the axoneme forming discrete cylindrical formations and the postal end of the axoneme extends uncovered outside of the mature sperm cell (Schwalm 1997).

The tail of Dipteran sperm cells is extremely elongated and the reason for this remains unknown. In some *Drosophila* types the tail of the sperm cells reaches a length of 58 mm, which is 20 times longer than the whole animal and 15000 times the length of the human sperm. Bundles of mature sperm are stored in a pair of seminal vesicles.

During copulation, sperm is deposited into the female spermatothecas through an extendable penis equipped with a sperm pump. Then, the sperm migrates from the spermatothecas to the seminal receptacle of the female, providing a lifelong supply of gametes for egg fertilization.

Spermatogenesis takes place during the whole adult life of the male fly (Ashburner and Wright, 1980). Although the first mature sperm cells are formed about 6 h after the eclosion of the adult male fly (Roberts, 1998), the newly eclosed males are not capable of successful copulation earlier than 12 h after eclosion. That is when the necessary amount of a protein called "sex peptide" is produced in the accessory glands of the newly eclosed males. This protein consisting of 36 amino-acids, accompanies mature sperm during copulation and it is necessary to stimulate oviposition in the female flies. The necessary amount of this protein is synthesised about 12 h after ecdysis, and only then are males considered sexually mature (King 1970; Stromnaes and Kvelland, 1962; Soller et

al, 1999; Panagopoulos et al 2004). Except of the stimulation of egg-laying, the presence of this protein within the female organism, makes the female unwilling for further copulation for at least 5 days (King, 1970; Soller et al, 1999; Ashburner and Wright, 1980).

Transplantation of accessory glands from males to virgin female flies stimulated oviposition. Oviposition was also stimulated when sterile mutant males but with healthy - operating accessory glands were mated to virgin females. However, transplantation of seminal vesicles or testes or fat body from males to virgin females did not stimulate oviposition (Engelmann, 1970).

Oogenesis in *Drosophila melanogaster* is a model biological system, very well studied with a good timing of its developmental stages under certain laboratory conditions, (King 1970; Horne-Badovinac and Bilder 2005; Bastock and Jonhston 2008; Panagopoulos et al. 2004). It starts at the last stages of pupation, a few hours before the eclosion of the adult female insect, and it is divided into 14 discrete developmental stages. Stages 1-7 are called provitellogenic (or preyolk). Stages 8-10 are called vitellogenic (during which accumulation of yolk proteins takes place in the oocyte), and stages 11-14 are called post-vitellogenic or choriogenic (during which there is further growth of the egg-chamber, and synthesis of the chorion by the follicle cells which surround the 15 nurse cells and the oocyte).

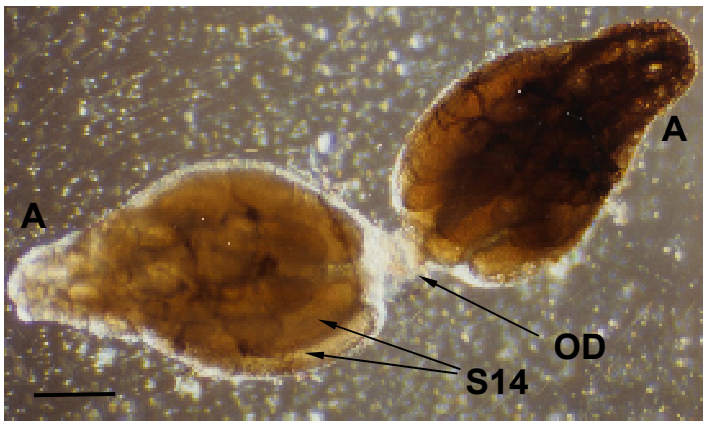

Figure 1. Photograph of a pair of ovaries of a sexually mature female fly 48 h after eclosion. Separate ovarioles and egg chambers are visible within the ovaries. Both ovaries are packed with mature eggs of stage 14. The arrows (S14) indicate two stage 14 mature eggs at the posterior ends of two adjacent ovarioles. The ovaries are connected between them at the posterior sides by the common oviduct (OD). A: anterior sides. Bar: 50 μm.

Each *Drosophila* female adult insect has two polytrophic ovaries (the nurse cells are enclosed together with the oocyte within the follicle) (Fig.1). Each ovary consists of 16 to 20 parallel ovarioles (Fig. 1, 2). Each ovariole is an individual egg assembly line, with new egg chambers in the anterior moving toward the posterior as they develop, through 14 successive stages until the mature egg reaches the oviduct. The posterior side of each ovary ends in a lateral oviduct, to which every ovariole of the ovary is connected. Then, the two lateral oviducts are joined to form a common oviduct (King 1970; Horne-Badovinac and Bilder 2005; Panagopoulos et al 2007a), (Fig. 1).

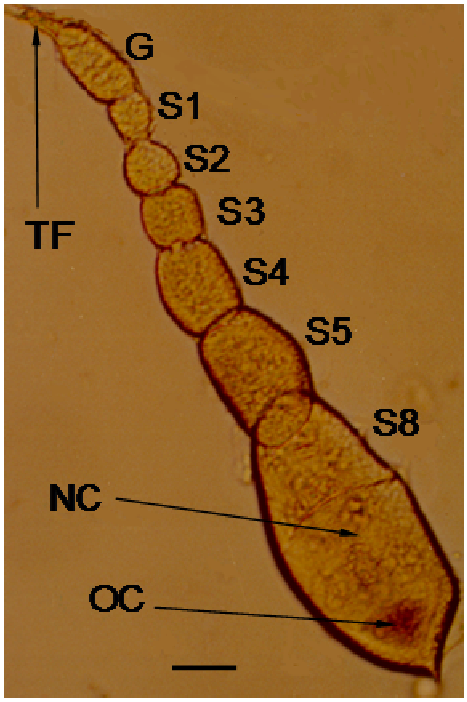

Figure 2. Photograph of Ovariole through optical microscope with clearly visible terminal filament (TF), germarium (G), and vitellarium consisting of stages 1-5 follicles and a stage 8 follicle. Bar: 10 μm.

The most anterior region of each ovariole is called the germarium (Fig. 2). At the anterior tip of the germarium right after the terminal filament (TF) there are two or three germ line stem cells. These produce mitotically active cells called cystoblasts or oogonia, each one of which undergoes four mitotic divisions to form a 16-cell cluster. Each egg chamber consists of the cluster of the 16 germ

cells surrounded by an epithelial monolayer of somatic follicle cells. The main region of the ovariole posterior to the germarium is called vitellarium (Fig. 2). The cyst of the 16 germ cells surrounded by the follicle cell monolayer, leaves the germarium to enter vitellarium as a stage-1 egg-chamber. After 14 successive developmental stages it becomes a mature egg about 48 h later. The 16 germ cells of the cyst are interconnected by cytoplasmic bridges known as "ring canals" or ring channels. Among the 16 germ cells there are eight with one ring canal, four with two, two with three and two with four ring canals (Bastock and Jonhston 2008). Of the two cells with four ring canals the one more distant from the anterior pole of the cyst differentiates to become the oocyte, the remaining 15 closer to the anterior becoming nurse cells. The oocyte is always one of the two cells of the first mitotic division of the cystoblast. The nurse cells enter a phase of endoreplication and become highly polyploid during the rest of oogenesis, synthesising cytoplasmic components and nutrients to be transported into the oocyte. Approximately 80 follicle cells surround the germline cyst at the time that it buds from the germarium as a stage 1 egg-chamber. The follicle cells divide mitotically until the end of stage 6, at which time they undergo three rounds of endoreplication and growth, amplifying chromosomal regions required for eggshell production. The oocyte remains arrested in prophase I until late stage 13, when the nuclear envelope breaks down, and meiosis progresses to metaphase I, where it remains arrested again during the final stage 14, until fertlization occurs (Panagopoulos et al 2007a).

The ovaries are connected through a network of vessels, spread over their entire surface, with the insect's fat body from which they get all necessary nutrients for the development of the egg-chambers. The fat body is a volume filled with hemolymph. Vitellins (major storage proteins produced by the fat body) are transported to the developing eggs through the hemolymph. The developing oocyte absorbs hemolymph (and vitellins) through the vessel network by pinocytosis (Schwalm 1997).

A pair of spermatothecas connected to the uterus and the gonopore is able to store enough sperm for the fertilization of all the mature eggs produced by the female insect during the whole adult life which usually lasts about 20 days.

A single copulation is usually enough for the females to get all the sperm necessary for their whole adult life. In case of a subsequent copulation, the newer sperm removes the older and takes its place within the spermatothecas and the seminal receptacle.

Around the follicle cells of the cyst there is a basic membrane made by a thin layer of connective tissue. Each ovariole is surrounded by an epithelial membrane,

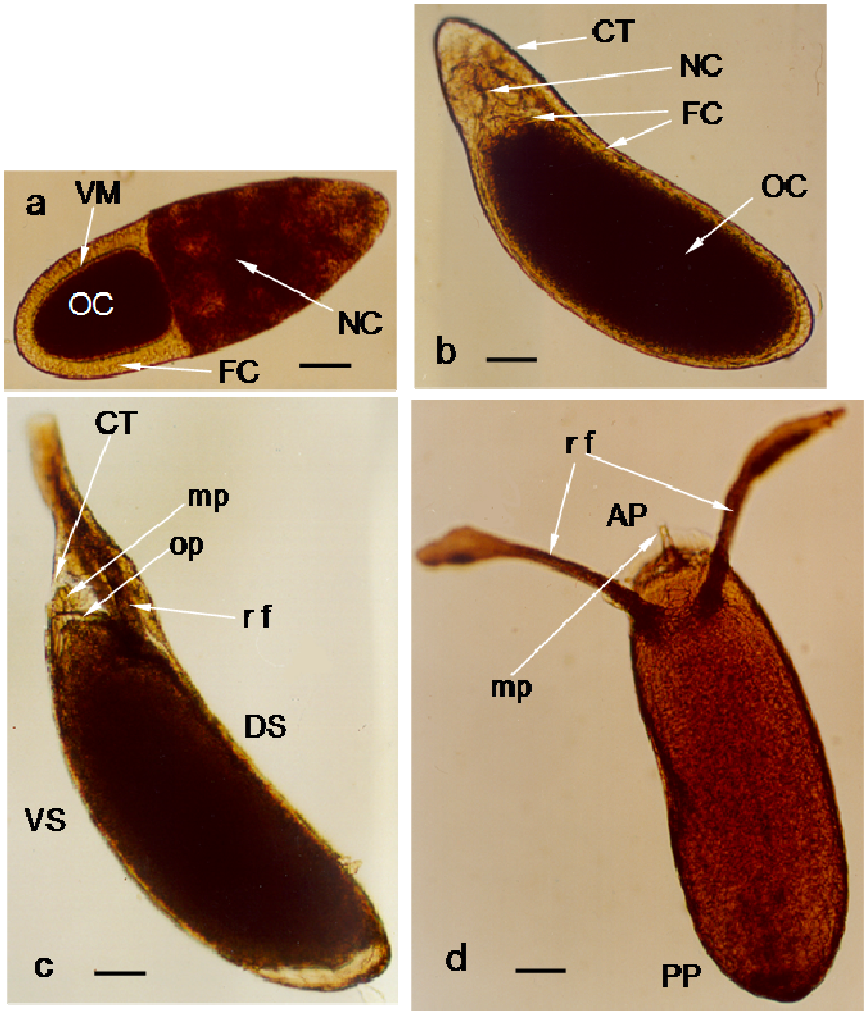

Figure 3. a) A stage 10 follicle. The oocyte (OC) occupies half of the egg's volume and it is surrounded by the follicle cells (FC) which have left the nurse cells (NC) outside of their cyst. Around the oocyte, the vitellin membrane (VM) is visible. b) A stage 12 follicle. The nurse cells (NC) are significantly shrunken as they undergo programmed cell death. The oocyte (OC) is increased in size absorbing the contents of the nurse cells. c) A stage 14 (mature) follicle, still surrounded by the thin membrane of connective tissue (CT). The oocyte has occupied the whole egg's volume. On the anterior pole the respiratory filaments (r f), the micropyle (mp) and between them at the anterior-dorsal side of the eggshell the operculum (op), are visible. Dorsal side (DS) and ventral side (VS) are distinguishable. d) Dorsal view of a laid - fertilized egg. Anterior pole (AP), posterior pole (PP). The respiratory filaments (r f) and the micropyle (mp) are clearly visible, [Bars: 10 μm].

called epithelial sheath. Then there is an outer membrane surrounding the whole cluster of ovarioles and enveloping the entire ovary, called peritoneal sheath.

While the first follicle is getting developed, the next follicles follow at earlier stages and they all develop simultaneously within the ovariole. When the first egg completes its development, it leaves the ovariole at the posterior end and enters the oviduct to get laid. Oogenesis, is called the whole process of egg development through the germarium and the 14 developmental stages within the vitellarium to become a mature egg ready to be fertilized and laid. The movement of the eggs towards the posterior of the ovariole during their development is accomplished by rhythmic contractions of the epithelial and peritoneal sheaths (King 1970).

During the provitellogenic stages (1-7) the eggs increase their size. All of the 16 germ cells increase their volume simultaneously absorbing nutrients by the hemolymph and synthesizing cytoplasmic organelles. The follicle cells increase in number by mitotic divisions from about 80 to about 1200, at which number they remain until the completion of oogenesis.

During the vitellogenic stages (8-10), considerable increase in the size of the egg takes place, mainly because of accumulation of yolk (vitellin) proteins in the cytoplasm of the oocyte and thus, considerable increase in its size. The vitellins or yolk proteins are produced by 3 initial proteins called vitellogenins which are simultaneously synthesized in the fat body and in the follicle cells, and transferred to the ovaries through the hemolymph. The oocyte absorbs them by pinocytosis through the extracellular space between adjacent follicle cells. During the vitellogenic stages, the follicle cells by proper movements leave the nurse cells outside of their cyst surrounding only the oocyte at stage 10 and produce around it a membrane called vitellin membrane. The whole follicle is still enclosed by the basic membrane made by connective tissue. At stage 10, the oocyte has already increased enough to occupy half of the volume of the whole egg-chamber (Fig. 3a). It is reported that the first vitellogenic follicles appear about 9 h after the adult ecdysis (Ashburner and Wright, 1980). According to our observations and the conditions of our laboratory, this takes place 10-15 h after the eclosion of the adult insect.

During the post-vitellogenic (also called choriogenic) stages 11-14, the egg increases even more in size due to further increase of the oocyte. The oocyte absorbs through the ring canals macromolecules and organelles from the nurse cells (lipids, mRNA ribosomes, etc). The size of the nurse cells consequently decreases, as they transfer their contents to the oocyte through the ring canals and undergo programmed cell death. Finally their remnants are engulfed and absorbed by phagocytosis by the adjacent follicle and epithelial cells. The follicle cells build a new membrane around the oocyte (and around the vitellin membrane they

Table 1. Developmental Stages of Drosophila melanogaster Oogenesis, with corresponding functions and durations, at 25 °C.

Stages of Oogenesis	Function	Duration
S1-S7 Provitellogenic	The 16 germ cells of the cyst budded from the germarium are simultaneously developed and increase their volumes. The follicle cells which form a monolayer surrounding the cyst, proliferate mitotically and increase their number from approx. 80 to approx. 1200 which they retain until the completion of oogenesis.	~ 20 h
S8-S10 Vitellogenic	The most distant from the germarium germ cell differentiates to become the oocyte and increases its size by absorbing yolk proteins though hemolymph until it occupies half of the egg's volume at stage 10. The follicle cells leave the nurse cells out of their cyst at stage 10 and build around the oocyte the vitellin membrane.	~ 22 h
S11-S14 Post-Vitellogenic	The oocyte increases even more occupying at stage 14 the whole egg volume by absorption of organelles and cytoplasmic contents from the nurse cells which undergo programmed cell death. The whole egg volume increases considerably. The follicle cells build the chorion with its special structures like the respiratory filaments and the micropyle around the vitellin membrane, engulf and absorb by phagocytosis the nurse cell remnants and finally they also undergo programmed cell death at stage 14	~ 6 h

produced before), called "chorion". Chorion is the most important protective part of the egg. After the completion of the chorion and the absorption of the nurse cell remnants, the follicle cells degenerate also by programmed cell death at stage 14.

While the mature egg leaves the ovariole to enter the oviduct, a mass of degenerating nurse and follicle cells is left behind at the end of the ovariole. The functions and durations of the stages of oogenesis are summarized in Table 1.

The structure of the chorion (which is the outer and hardest layer of the egg-shell) consists of a) wax layer in contact with the vitellin membrane, b) the inner chorionic zone, c) the endochorion, (consisting of floor, columns and roof with cavities filled with soft material) and, d) the exochorion (the outer layer). (Margaritis et al, 1980).

The fully developed (mature) egg (without the respiratory filaments) is approximately 100 μm long and 40 μm wide, with distinct anterior, posterior, dorsal and ventral sides (fig. 3c, d). At the anterior pole of the chorion a micropylar cone forms containing a tubular canal for the entrance of the sperm and fertilization of the egg, called micropyle.

The two respiratory filaments are chorionic appendages at the anterior-dorsal side of the egg-shell, secreted by the follicle cells during stages 12 and 13, serving as twin breathing tubes on occasions when the embryo is submerged. When the embryo is covered with water, the respiratory filaments extract oxygen from it (Hinton 1969). Between the respiratory filaments and the micropyle there is a circular structure surrounded by collar, called operculum. This is the part of the egg-shell that breaks open for the larva to get hatched after the completion of embryogenesis. On the surface of the chorion of a mature-laid egg, one can easily see under the microscope the hexagonal prints of the detached follicle cells.

When a mature egg enters the oviduct, contractions of the oviduct push the egg into the uterus where fertilization takes place. As the egg passes through the vagina to get released, its micropyle becomes opposed to the opening of a duct that transforms sperm cells from the seminal receptacle. A necessary amount of sperm is inserted into the mature egg through the micropyle to activate the egg. The egg continues its way through the vulva (ovipositor) and gets laid. Therefore, insemination of the egg occurs just a few minutes before oviposition. Immediately after insemination, cytoplasmic dislocations take place in the oocyte while its nucleus completes the second meiotic division. At the same time the sperm nucleus decondenses its chromatin to form chromosomes. Both nuclei undergo an S phase while coming closer to each other and get fused to form the zygote, (Schwalm 1997). The eggs are released through the vagina and its external part the vulva, (the orifice through which copulation takes place).

Our studies related to *Drosophila* oogenesis (Panagopoulos et al. 2004; 2007a; 2007b; 2010; Panagopoulos and Margaritis 2003) have shown that there is some control mechanism in *Drosophila* oogenesis that, once the first egg-chamber of each ovariole enters the vitellogenic stages (S8), the development of the

following egg-chambers is arrested at the provitellogenic stages, until the first one proceeds to the post-vitellogenic. Then, while the first one is at the post-vitellogenic stages, the following one remains still arrested at the provitellogenic or proceeds slowly to early vitellogenesis (S8) until the first one is fully matured and laid. For this, in each ovariole, one very rarely sees two follicles developed beyond more than stage 8. This observation of ours is still today not fully verified by other experimenters. King has reported that rarely can be seen more than one mature egg in each ovariole, and also that, "some control mechanism" causes a cessation of egg production, once the ovarioles are packed with mature eggs (King 1970). Since most of the volume of an egg-chamber is attained during mid-late vitellogenic and post-vitellogenic stages S9-S14, by a considerable amount of yolk proteins transferred from the insect's fat body to the oocyte during stages S9-S10, and the synthesis of different biomolecules, the chorion, etc., during the post-vitellogenic stages plus the transfer of the nurse cells' cytoplasmic contents to the oocyte, the size of the ovary depends basically on how many ovarioles contain an egg (the first one) at the post-vitellogenic stages S11-S14.

Gametogenesis and consequently reproductive capacity are useful markers to assess the biological activity of external stress factors. Any effects on gametogenesis will lead to changes in oviposition and reproductive capacity (fertility).

Embryonic Development

Within minutes after fertilization and oviposition, the zygote nucleus divides mitotically in rapid synchronous cycles retaining a common cytoplasm. During this phase each nucleus is surrounded by small amount of microtubule-enriched cytoplasm distinct from the yolky components of the egg's cytoplasm. Each nucleus with its amount of microtubule-enriched cytoplasm is called, an energid. Two hours later, more than 500 energids are present, migrating to the periphery of the egg. They continue to proliferate and get evenly distributed on the egg's surface, forming a so-called syncytial blastoderm. Then each energid on the egg's surface builds a plasma membrane around it and becomes cellularized. The cellularization of the blastoderm occurs during the 14^{th} cell division cycle, about 5 h after fertilization.

The energids gathered at the periphery cause the cell membrane of the oocyte to bulge outward, creating "buds" on the surface of the chorion. Membrane folds originate between the buds after the 13^{th} cycle and extend centripetally like

furrows into the egg. As soon as the advancing furrows reach the yolky interior, they expand laterally underneath each blastoderm cell forming a membrane covering the yolky interior of the egg.

Heterochronous cell proliferation and subsequent aggregation of cells along the ventral side of the egg, lead to the formation of a single layer of columnar cells which is called germ band and constitutes the embryonic primordium from which all the parts of the insect's body will be formed. Meanwhile, during the 10^{th} division cycle of the energids, about 2 hours after fertilization and 3 hours before the cellularization of the blastoderm, some energids at the posterior pole behave differently from the rest energids of the blastoderm, push out of the egg surface and segregate from it becoming cellularized. These pole cells will constitute the part of the germ band from which, during organogenesis, the gonads will be developed.

After the cellularization of the blastoderm and the germ band formation, organogenesis starts. Cells along the ventral axis of the egg's surface invaginate into the yolk creating a ventral furrow which then closes quickly, forming a mesodermal tube which will become the insect's midgut. The posterior end of this tube will form the proctodeal invagination. At the same time the germ band extends rapidly around the posterior pole, and the proctodeal invagination with the pole cells move toward the anterior along the dorsal midline of the egg's surface, until they have covered the 2/3 of the distance to the anterior pole. The pole cells follow the deeping invagination, penetrate its outer layers and become surrounded by mesodermal gonad components, (Schwalm, 1997). During the germ band extension, external delineations become apparent on the embryo surface.

About 5-8 h after fertilization, a network of respiratory microtubes (tracheal pits) begins to develop at the posterior regions around the mesodermal tube. Then the germ band retracts and the midgut forms rudiments.

The part of the germ band which covers the ventral surface and lateral regions of the blastoderm that join along the ventral midline, is a neurogenic region of the ectoderm that generates neuroblasts and produces ventral ganglia about 10 h after fertilization. From these cells the central nervous axon will be developed. Genes that control which cells will differentiate into neural, have been identified in *Drosophila* (Doe 1992). Anterior segments are retracted into the thorax segments and the head begins to appear. At the same time the ganglia of the central nervous system are getting developed. As the buds of the legs, antennae and other sensory projections appear, neurons from the corresponding regions of the epidermis extend towards the central nervous system. At the anterior pole, three neurogenic regions seem to be responsible for the creation of the head and the optical lobes. After the first embryonic stages the head gets covered and disappears under other

developing parts of the organism and appears again at the late post-embryonic stages during pupation (Schwalm 1997).

During embryogenesis starts the development of specific disc-shaped formations close to the surface that are called imaginal discs. These are histoblasts from which the formation of all the external features of the insect will begin during post-embryonic development (larval stages). The function of each imaginal disc will depend on its position along the anterior-posterior and the dorsal-ventral axis. After the formation of the primordial of each disc, their cells rarely divide during the rest of embryogenesis but they will begin to proliferate during larval development.

Embryogenesis in *Drosophila melanogaster* lasts about 24h, at 25°C, (from fertilization and oviposition of the mature egg to the hatching of the first instar larva).

Post-Embryonic Development, Metamorphosis, Life Cycle, Oviposition

The insect's morphological changes during post-embryonic development are described by the general term, metamorphosis. Metamorphosis in all insects is controlled by 3 hormones; The encephalic or prothoracotropic hormone (PTTH), the ecdysone (or molting hormone), and the juvenile hormone (JH). The post-embryonic development comprises the 3 larval stages (called 1^{st}, 2^{nd} and 3^{rd} instars), and the pupal stage.

As written already, about 24h after fertilization and oviposition, the embryo hatches from the egg as a 1^{st} instar larva. Larvae have the appearance of a worm which is initially transparent and becomes white during the first day after hatching. Each of the three larval instars is terminated by ecdysis (shedding of the exoskeleton). Each next instar is increased in size. The significant increase of the larval size during its development through the three instars, is mainly due to a significant increase in cell size rather than number. Larval cells become polyploid by repeated DNA replication without division, producing parallel copies of each chromosome which lie side-by-side forming "giant" polytene chromosomes. These can be easily observed in the salivary gland cells of the 3^{rd} instar larvae after dissection - extraction of the glands, staining and squashing. The only cells that proliferate within the larval body are the cells of the imaginal discs during the third larval instar which will become the cells of the adult insect. The rest of the larval cells finally undergo autolysis during the whole process of metamorphosis.

Each ecdysis is initiated by PTTH produced by neural secretory cells in the brain and released from corpora cardiaca. This hormone triggers the molting (prothoracic) glands to release ecdysone in the hemolymph. Ecdysone induces "puffing" of selected chromosome regions that include epidermis genes and thus, induces transcription of these genes and protein synthesis that triggers the shedding of the older skin by the new underlying one. The older skin breaks under the pressure of the hemolymph and ventral muscle contractions. The new skin increases by mitotic cell divisions. The whole process is termed apolysis. JH released by the corpora allata suspends ecdysis, restricting the insect's development within each specific stage before passing to the next.

The three larval instars last about 4 days (at 25°C). During the first two instars lasting one day each, the larva is within its food feeding itself constantly, while at the end of the third instar which lasts two days, it comes onto the surface. For populations bred in the laboratory within vials, at the end of the third instar, larvae leave their food and climb on the walls of the glass vials a few mm or even a few cm above the surface of the food where they remain still, preparing for the next (pupal) developmental stage.

The embryonic (imaginal) discs under the larval epidermis are mainly developed during the third larval instar. There are 19 imaginal discs, each one responsible for the development of certain parts of the adult insect's body. The function of each disc is predetermined depending on its position in the larval body. This is shown by experiments transplanting one disc into another's position. The transplanted disc differentiates autonomously from the adjacent cells and creates tissue according to its original and not its new position. This memory property of the imaginal disc cells related to position information is due to certain genes called homeotic selector genes (HOM genes). These genes are the same that play a key role in patterning the body parts of other animals including human. Thus, the set of imaginal discs from which the adult fly will develop, carry stored positional information.

Following the larval instars, the post-embryonic insect becomes a pupa or chrysalis. The pupal stage of development lasts about four days under normal conditions (25°C). The third larval mold and puparium formation takes place at 120 h and eclosion (ecdysis) of the adult fly 216 h after fertilization – oviposition. During the 4 days of the pupal stage the insect remains immobilized outside of the food, covered by a semitransparent hard skin (puparium). Pupation is signaled by the emersion of the cephalic complex and during this stage all the external parts of the adult fly are formed by the imaginal cells which do not divide anymore but only grow and differentiate (Roberts 1998). At the end of the pupal stage the production of juvenile hormone stops and the final metamorphosis into the adult

insect takes place. During the last hours before eclosion the pupae become black and the red color of the eyes of the adult insect becomes visible through the puparium. In the laboratory, this intense dark color of the pupae is the sign that newly emerged adult flies are going to be eclosed within the next few hours in order to collect them for experiments.

The total time between fertilization and the adult ecdysis is thus nine days normally at 25°C. The above described phases of *Drosophila melanogaster* metamorphosis from fertilization to adult fly and the time points of their occurrence after fertilization, at 25°C, are shown in Table 2.

Table 2. Life cycle of Drosophila melanogaster and life-phase durations.

Life Phase	Duration
Oogenesis	48 h
Fertilized egg, (Embryogenesis)	24 h
1st instar Larva	1 day
2nd instar Larva	1 day
3rd instar Larva	2 days
Pupa	4 days
Adult fly	20-25 days

When the adult fly ecloses from the puparium its wings are significantly shorter than its body's length, soft and wrinkled. Its body is thin and long after the shape of the puparium and almost white except for the eyes. All other colors are very dim. From the first minutes after eclosion the hues of all parts of the body become more and more intense, the wings unfold gradually becoming longer and stronger and within the first hour the adult morphology is completed.

Male adult flies are sexually mature and ready for successful copulation about 12h after eclosion, while females about 45 h after eclosion when oogenesis is completed for the first eggs within the ovarioles and the first mature eggs are ready to be fertilized and laid. Newly eclosed and synchronized male and female flies put together in the same vial, start sexual activity by the end of the second day after eclosion. During copulation which lasts about 10 min, the male leaves sperm plus other substances synthetized by its accessory glands including the special protein, sex-peptide.

Normal temperature for the insect is 18-29°C while the optimum temperature is 24-26°C. Optimum relative humidity is 70-80%. The duration of the insect's adult life is usually 20-25 days as already mentioned.

As explained before, oviposition in adult female *Drosophila* is enhanced by the protein sex-peptide which is produced by the male accessory glands and accompanies the sperm. Females lay their eggs within their food. The respiratory filaments on the anterior-dorsal side of the egg remain outside so that the embryo can breathe. Oviposition of inseminated females is at its maximum between the 3rd and the 6th day of adult life. Then it slightly decreases but remains close to the maximum for the next 6 days. After the 12th day, oviposition decreases considerably and after the 20th day usually stops as the fly is reaching the end of its life-cycle (Panagopoulos et al 2004; Bos and Boerema 1981; Shorrocks 1972). Oviposition strongly depends on environmental factors such as temperature, atmospheric pressure, humidity, food availability and quality, chemicals, radiation, oviposition sites, etc. (King 1970; Srivastava and Singh 1998; Gruntenko et al 1996; Levengood and Shinkle 1960; Panagopoulos et al. 2004; Nezis et al. 2000; 2002; Drummond-Barbosa and Spradling 2001; McCall 2004). During the last 5 days of their lives the flies are considered to be old. The old females continue to lay eggs most of which do not develop. In other words, eggs laid during the last days of the fly's life cycle exhibit a large percent of mortality. In contrast, eggs laid by newly eclosed flies, during the first days of their adult life have a statistically non-significant percent of mortality, smaller than the standard deviation of the insect's daily oviposition. (Panagopoulos et al. 2004).

Relation between Insects and Mammals

In the previous paragraphs we attempted a brief general description of the insect's life cycle emphasizing the early phases (gametogenesis, embryonic and post-embryonic). In the following paragraphs we shall attempt to explain why experiments with this model experimental animal can be extremely useful for fast estimates of possible environmental influences on humans.

Comparison of gene sequences between different animals has revealed that a large proportion of *Drosophila* as well as others insects' genes, have homologues in vertebrates including human and the other mammals. These are the HOM gene complex mentioned above, that define compartments of the body in insects. The HOM genes have been highly conserved in evolution so that homologues in mammals called Hox genes can be found. Thus, it is considered that insects and mammals come from a common ancestor in whom a single primordial homeotic selector gene underwent repeated duplication to form a series of such genes that eventually formed what now is called, a HOM complex. Then, in the lineage that

led to mammals this complex was repeatedly duplicated again to form the four Hox complexes that exist in all mammals including humans (Alberts et al 1994). In addition to the astonishing gene similarities between insects and mammals, the basic cellular processes are identical in insect and mammalian cells.

In contrast to these similarities at the cellular level, insects including *Drosophila*, are much more resistant to certain types of environmental stress, such as radiation, than mammals. In all the experiments performed so far to compare the vulnerability to different types of radiation between insects and mammals, insects are found to be tens or hundreds of times more resistant than mammals, (Koval and Kazmar 1988, Koval et al 1979, 1977, Abrahamson et al 1973). Therefore, it is very reasonable to assume that an effect caused by radiation on *Drosophila* can be expected to be caused also in the human organism. The great advantage in studying this effect on *Drosophila* instead of studying it on mammals, is that it can be observed within a few hours after exposure while in mammals (including humans) it will be observed usually months or years after the exposure to the same type of radiation.

In contrast to radiation, insects can be easily killed by certain types of chemicals (like DDT) that do not seem to have an immediate significant effect on mammals. But then again, we must consider the fact that any deleterious effect that would take months to have a fatal effect on small mammals and several years on humans could be observed within a few days or even hours in insects and particularly in *Drosophila*.

Still, radiation seems to be the most serious threat for human health in our modern environment considering the numerous types of antennas, power lines, personal and home devices like mobile phones, microwave ovens, Wi-Fi, etc. Therefore, proper experimental protocols regarding environmental radiation effects on *Drosophila* can be very useful.

Due to the higher tolerance of *Drosophila* to radiation compared to mammals, in combination with the much shorter biological cycles and the similarity of cellular functions, *Drosophila* can be used as a fast *in vivo* indicator of environmental radiation bioactivity and genotoxicity.

Earlier Developmental Stages are more Vulnerable to Stress than Later Stages

From the above description we can see that especially gametogenesis and embryogenesis are far more sensitive than later developmental stages in this

particular insect and similarly in all animals. It is amazing how many concerted precise functions lead to the formation of the adult organism. The more initial a function is, the more sensitive to exogenous influences. For this it is well known that in mammals the early embryonic stages are more sensitive to ionizing radiation than the following stages of embryonic development (Coggle 1983; Nias 1998). In mammalian gametogenesis it is well known that oogonia and even more the stem cells from which they come from, are more sensitive to ionizing radiation than the oocyte and the follicle at later stages of oogenesis. Similarly, spermatogonia and even more the corresponding stem cells are more sensitive than mature sperm cells. Damage of the stem cells which produce the gametes can cause permanent sterility of the animal.

Thus, reproductive cells from which the future organism will be developed, seem to be more sensitive to stress than other types of cells. This is, at least in part, due to the fact that reproductive cells undergo multiple biological processes like mitotic and meiotic divisions, synthesis of all kinds of biomolecules, etc. This fact is in line with the old empirical law of Bergonie and Tribondeau according to which proliferating cells and tissues are more vulnerable to radiation than non-proliferating ones and also that the less differentiated cells are more radiosensitive than highly differentiated ones (Bergonie and Tribondeau 1906; Prasad 1995).

Fortunately, germ stem cells, oogonia and spermatogonia although more sensitive, are fewer in number, smaller, and much more protected by the surrounding cells and tissues than the more differentiated and developed follicular and sperm cells. This seems to be the reason why exposure to so many different kinds of genotoxic agents like radiation, EMFs, chemicals and other environmental threats have not caused already the disappearance or deformation of life on our planet.

In our experiments regarding non-ionizing radiation and electromagnetic fields (EMFs) effects on *Drosophila melanogaster*, it was shown that gametogenesis was a more sensitive developmental stage than embryogenesis. Exposure of developing follicles during early and mid-oogenesis resulted in elimination of large numbers of them and corresponding decrease in oviposition, while exposure of fertilized embryos (eggs) to the same radiation/fields did not cause a significant effect (Panagopoulos and Margaritis 2003; Panagopoulos et al 2004).

Additionally, oogenesis is more easily studied than spermatogenesis due to the easier access anatomically, the larger size of the ovaries and individual follicles than the corresponding sizes of the testes and the sperm cells, and the simultaneous existence of egg-chambers at many different developmental stages within the ovaries which can be easily treated by different laboratory techniques.

For these reasons we focused on oogenesis for the study of the biological effects of radiation and EMFs.

Due to the high sensitivity of oogenesis to exogenous stress factors and the existence of special check-points during early and mid-oogenesis (most sensitive developmental stages), all the abnormally developing eggs are properly eliminated by stress-induced cell death during the provitellogenic and vitellogenic stages, and thus, usually, exogenous stress factors do not have an effect on the offspring's genome and morphology but rather on the number of laid eggs (oviposition) and, consequently, on the number of developing post-embryos (larvae and pupae).

In addition, when the parental flies are young (newly eclosed), during the first days of their adult lives – at least until the eighth day – the fertilized eggs laid by them as well as the larvae and pupae derived from them do not have any significant mortality, and thus, the number of F_1 pupae coincides with the number of fertilized eggs. For the same reason this number coincides also with the number of F_1 eclosed flies (offspring).

This is why the number of F_1 pupae at certain time-points after the eclosion of the newborn maternal flies, is a representative measure of the insect's reproductive capacity and a reliable indicator for the biological activity of any exogenous factors.

Thus, the biological activity of different environmental factors may be assessed by their effect on *Drosophila* oviposition, (changes in the number of laid eggs), reflecting effects on gametogenesis. The effect on oviposition can in turn be assessed by the number of F_1 pupae.

The use of *Drosophila melanogaster* oviposition for the assessment of the biological activity of man-made electromagnetic fields is described in the following sections.

Man-Made Non-Ionizing Electromagnetic Radiation as a Main Factor of Modern Environmental Stress for Living Organisms

Radiation has always been one of the main stress factors for all living organisms in the terrestrial environment. While non-ionizing natural electromagnetic radiation from the sun and the stars (infrared, visible light and weak ultraviolet) has always been necessary for almost any form of life on our planet, natural ionizing electromagnetic radiation from vacuum ultraviolet to gamma rays as well as particle radio-energy (α, β, mesons, etc) emitted mainly

from natural minerals and our cosmic environment, although catastrophic in large doses has always been present at relatively small doses and living organisms had adapted to its presence. The appearance of man-made ionizing radiation in the form of x-rays and artificial radio-energy (e.g. artificial radio-nuclides and radiations produced by nuclear reactors or accelerators or nuclear weapons) at the end of the nineteenth and with the onset of the twentieth century, was lethal for its inventors who didn't really know the dangers of their inventions (considering e.g. that Roentgen, Marie and Irene Curie died from different types of cancer). When man-made ionizing radiation is restricted to well-controlled medical and scientific applications its lethal action would not affect the general population and can be used in small doses to improve human health. The great danger comes from the military and power production uses like atomic bombs and nuclear reactors. Soon after its invention, man-made ionizing radiation was known for its catastrophic power able to cause cancer and inherited mutations to all exposed living organisms with its effects lasting for decades and even more (Hiroshima, Nagasaki, Bikini islands, Chernobyl and, most unfortunately, during these very days Fukushima, just to mention a few).

Yet, another form of man-made radiation appeared also with the onset of the 20^{th} century: The non-ionizing electromagnetic in the form of polarized electric and magnetic fields associated with electric power transfer lines or information carrying electromagnetic waves (radio, television, mobile telecommunications, Wi-Fi, etc). The main differences from all previous types of electromagnetic radiation are that: a) it is not photonic (it is not transmitted in the form of small discrete packets of energy called photons which are naturally emitted by subatomic-submolecular events, but it can be transmitted in the form of long continuous or pulsed waves as it is emitted by electric oscillation circuits) and b) it is polarized, meaning that it can induce additive oscillations on any electric charge within living tissue, like for example the free ions which play a vital role within all living cells. This radiation cannot directly break chemical bonds or detach electrons from atoms or molecules and for this it is called "non-ionizing".

The levels of this type of radiation have increased dramatically during the last decades and continue to increase uncontrollably in exponential rates. While living organisms were adapted for millions of years to the previous types of radiation, they are suddenly surrounded by a milder but unknown throughout their development type of radiation at constantly increasing levels. While other types of stress factors like heat shock, chemicals, food deprivation etc. although potentially lethal in large doses were also known to living organisms - most of them since the beginning of their existence - non-ionizing man-made electromagnetic radiation was unknown. This radiation, especially at the extremely low frequency (ELF)

and microwave-radio frequency (RF) regions of the electromagnetic spectrum - which are the most frequently used - constitute the main type of modern environmental stress for all living organisms on planet earth (Panagopoulos 2011a; 2011b).

Living organisms seem to sense EMF exposure as environmental stress similar to heat (which is also electromagnetic radiation – usually infrared – but photonic and non-polarized).

Thermotolerance in *Drosophila* is accomplished by synthesis of heat shock proteins (HSP). These proteins protect cells from excessive heat by maintaining the structure and function of the other proteins within the cells.

As living organisms do no have special genes to protect themselves against man-made EMF exposure, it seems that in response to this exposure they activate heat shock genes. *In vivo* exposure of *Drosophila melanogaster* newly eclosed parental flies with their progeny eggs, larvae and pupae for 1 h twice each day for a total of 10 days to GSM 1900 MHz radiation from a mobile phone, induced very fast (within minutes) the synthesis of heat shock protein hsp70 in the F_1 larvae. The rapid induction of hsp70 synthesis by exposure to non-thermal levels of pulsed microwave radiation (about 14 orders of magnitude lower than thermal stimulus) showed that cells exhibit far greater sensitivity to EMFs than for heat (Weisbrot et al 2003).

The use of Drosophila Reproduction for the Assessment of the Biological Activity of Modern Environmental Stress

For the study of the biological activity of non-ionizing radiation we used *Drosophila melanogaster* flies, Oregon R, wild-type, held in glass bottles and kept in incubator at 25 °C, with 12-h periods of light and darkness and 70% relative humidity.

Drosophila melanogaster food was prepared with 450 ml water, 4g agar, 13g yeast, 32g rice flour, 16g sugar, 25g tomato pulp. The mixture was boiled for over 10 min to ensure sterility, which is preserved by the addition of 2 ml propionic acid and 2 ml ethanol. This food quantity is enough for 25-30 standard laboratory 50-ml cylindrical glass vials (tubes), with 2.5 cm diameter and 10 cm height, with equal quantity of food forming a smooth plane surface 1-cm thick at the bottom of each vial. The glass vials were sterilized before the food was added.

We studied effects of different kinds of electromagnetic fields (EMFs) on the reproduction of *Drosophila melangaster*. Specific experimental protocols were designed, based on the good timing of gametogenesis, embryonic and post-embryonic development of the insect as described above. The basic experimental procedure was as follows:

In each experiment, we collected newly emerged adult flies from the stock; anesthetized them very lightly with diethyl ether and separated males from females. We put the collected flies in groups of ten in the standard glass vials with food. The glass vials were closed with cotton plugs. The exposures to the different kinds of radiation/fields started at the first day ~1h after the insects were divided into the different groups and were awaken from the anesthesia.

In each group we kept the ten males and the ten females for the first 48 h of the experiment in separate glass vials. Keeping males separately from females for the first 48 h of the experiment ensures that the flies are in complete sexual maturity and ready for immediate mating and laying of fertilized eggs, (Panagopoulos et al. 2004).

After the first 48 h of each experiment, the males and females of each group were put together (ten pairs) into another glass tube with fresh food, allowed to mate and lay eggs for 72 h. During these three days, the daily egg production is at its maximum.

Five days (120 h) after the beginning of each experiment the flies were removed from the glass vials and the vials were maintained in the culture room for at least six additional days, without any further exposure. The removed maternal flies were collected and their ovaries were dissected and treated for different biochemical assays (such as TUNEL, rhodamine-conjugated phalloidin staining, etc - see below).

After the last six days, most F_1 embryos (deriving from the laid eggs) were at the stage of pupation, where they could be clearly seen with bare eyes and easily counted on the walls of the glass tubes.

As explained, this number of F_1 pupae is a representative estimate of the insect's oviposition and reproductive capacity. In the next paragraphs when we refer to the insect's reproductive capacity we mean the number of F_1 pupae.

The exposures to the different types of radiation-EMFs always started at the first day (day of eclosion of the adult flies) of each experiment, and lasted for a total of five or six days.

The temperature during the exposures was monitored within the vials and within the mass of the food by a mercury thermometer with an accuracy of 0.05°C (Panagopoulos et al. 2004).

We exposed the insects to one of the following types of electromagnetic radiation-fields: a) GSM 900 MHz pulsed microwave field-radiation from mobile phones at intensity range 1-400 μW/cm^2, b) GSM 1800 MHz pulsed microwave field-radiation from mobile phones 1-300 μW/cm^2, c) Extremely Low Frequency (ELF) - 50 Hz sinusoidal magnetic filed, 1-70 G, d) Very Low Frequency (VLF) – 10 kHz sinusoidal damping electric field produced in pulses at a repetition frequency of 50 Hz (ELF), 1-10 kV/cm.

The daily duration of exposure to the microwave fields was 6 min per day (for the five days as described above). The exposure to the ELF magnetic field was constant for the five days, and the exposure to the ELF-VLF pulsed electric field was half an hour every two hours constantly during the five days (Panagopoulos and Margaritis 2003; 2008; Panagopoulos et al 2007a; 2007b; 2010; Panagopoulos 2011a; 2011b).

The experiments showed: a) A decrease 2-10% in the reproductive capacity, following exposure to the alternating magnetic field. The effect increased with increasing magnetic field intensity. b) A large increase 20-40% in the reproductive capacity, following exposure to the pulsed electric field. The effect increased with increasing electric field intensity and c) a very large decrease in the reproductive capacity, up to 60%, following exposure to the GSM 900 and 1800 MHz fields (Panagopoulos and Margaritis 2003; Panagopoulos 2011a; 2011b).

All fields significantly affected the reproductive capacity of both males and females. While the particular VLF pulsed electric field enhanced reproduction and did not seem to be genotoxic, the decrease in reproduction induced by the microwave and the ELF magnetic fields was found to be due to cell death induction in the reproductive cells (gametes) after DNA damage (see below).

The fact that all four types of radiation-fields tested with the use of the above described experimental protocol were found to have a significant biological action, shows that this experimental protocol based on *Drosophila* reproduction is a very sensitive and fast tool for the assessment of environmental bioactivity, and in particular for radiation bioactivity.

No temperature increases were observed during the exposures in our experiments within the vials containing the insects or within the mass of the food. Thus, the reported effects by all the types of radiation/EMFs tested, were found to be non-thermal.

Drosophila reproduction was also used before by other experimenters for the assessment of bioactivity of EMFs (Levengood and Shinkle 1960; Pay et a; 1978; Ramirez et al 1983; Ma and Chu 1993; Koana et al 1995) or other environmental factors like temperature (Srivastava and Singh 1998; Gruntenko et al 1996),

barometric pressure (Levengood and Shinkle 1960), food availability, chemicals, etc (McCall 2004).

In regard to temperature effects on *Drosophila* oviposition, it was found by other experimenters that four species of the *melanogaster* group: (*D. ananassae*, *D. bipectinata*, *D. malerkotliana* and *D. biarmipes*) lay significantly lower number of eggs at 19°C, than at 24°C or 30°C (Srivastava and Singh 1998). In some other experiments, heat stress – above 30°C – in *Drosophila virilis* and *Drosophila melanogaster* resulted in delay and decrease of oviposition accompanied by decreased JH hydrolysis (metabolism) in both species (Gruntenko et al. 1996). Thus, both low and elevated temperature are found to decrease oviposition in *Drosophila*. At the same time, a warmer environment within the insect's normal temperature variations (18-29°C), enhances oviposition (Panagopoulos and Margaritis 2003).

An innovation introduced by our experiments which brought a significant improvement in assessing the reproductive capacity (fertility) of this animal was that, instead of measuring the offspring (number of F_1 flies) which would increase the duration of each experiment by several days, we measured oviposition (number of laid eggs) by newly eclosed parental flies. Moreover, instead of assessing oviposition by counting the laid eggs under the microscope – a process that encounters considerable error - we assessed that by counting the number of F_1 pupae since, as already explained, this number coincides with the number of eggs when laid by newly eclosed insects during the first days of their adult lives (Panagopoulos et al. 2004; 2007a; 2007b; 2010; Panagopoulos and Margaritis 2010). The F_1 pupae can be easily counted on the walls of the glass vials without any error. This number under the conditions of our experiments coincides to the number of laid eggs (oviposition), since there is no statistically significant mortality of eggs, larvae or pupae that come from newly emerged paternal-maternal flies during their first 6 days of adult life. These innovations introduced by us made the assessment of reproductive capacity faster, easier and accurate.

In earlier works, performed to study the effects of magnetic fields (Ma and Chu, 1993, Ramirez et al, 1983) or RF fields (Pay et al, 1978), or temperature (Srivastava and Singh 1998), on the reproduction of the same insect, the procedures were based on the counting of laid eggs, an operation that encounters significant error as we explained. In addition, in some of those works, (e.g. Ma and Chu, 1993), they did not use newly emerged paternal-maternal flies, but instead, flies that were taken from the general stock population. This introduces uncertainty since eggs from older flies have a considerable percentage of mortality. In another of those works (Ramirez et al, 1983) only the female flies were exposed which were 4 days old and already mated when placed in the field.

Therefore, any effect on spermatogenesis or on mating was excluded and any effect on oogenesis was diminished. In the last of the above experiments (Pay et al, 1978), they studied the oviposition of individual pairs of adult flies that were developed from pupae that were exposed for only 10 min in a very intense microwave field (able to produce large temperature increases) 100 hours post-hatching. Therefore, the exposure took place many hours before the beginning of oogenesis, which as explained starts during the last stages of pupation. Besides, oviposition from individual pairs may have a large variability since some male flies do not accomplish copulation while others accomplish copulation with more than one female. In all the above experiments, a large number of non-developed laid eggs was reported both in exposed and control groups.

By introducing the above innovations in the way we assessed reproductive capacity/oviposition, the errors were minimized as well as the number of non-developed laid eggs.

Although the above described experimental protocol for the assessment of bioactivity was used in our experiments in regard to non-ionizing radiation/EMFs, it can also be used for the bioactivity assessment of other environmental factors like heat, humidity, barometric pressure, chemicals, etc.

In another series of experiments we have shown that the decreased reproduction, after exposure to the microwave fields as described above, is accompanied by decreased ovarian development during the developmental period of the first eggs in the ovaries, which starts at the late stages of pupation and lasts until about 48 h after the eclosion for the virgin female adult flies. To demonstrate this, we photographed under microscope and compared the size of intact ovaries between exposed and sham-exposed virgin female flies at certain time-points corresponding to the different stages of oogenesis of the first follicles, during the first 51 h after eclosion. This study showed that the ovarian size of the exposed insects is significantly smaller than that of the corresponding sham-exposed insects, (Fig. 4). [In the following section we describe that the decreased oviposition and the reduced ovarian development is due to the destruction of egg-chambers by the GSM radiation after cell death induction on their constituent cells]. The difference in the ovarian size between sham-exposed and exposed virgin female flies became most evident 39-45 h after eclosion when the first eggs within the ovaries were at the late vitellogenic and post-vitellogenic stages (mid - late oogenesis) during which they attain most of their volume. More than 45 h after eclosion, the difference in ovarian size decreased, as the first mature eggs of the sham-exposed insects were leaving the ovaries to get laid.

Similar significant reduction in the size of the ovaries is reported by other experimenters in nutrient-deprived flies, compared to flies conditioned to food

supplement with yeast. In that case, the reason for the reduced ovarian development was cell death induction at the mid-oogenesis checkpoint, due to the food deprivation (Drummond-Barbosa and Spradling 2001). Mutant flies in regard to abnormal insulin pathways regulation are also found to have decreased ovarian size, due to failure of the egg-chambers to develop to post-vitellogenic (choriogenic) stages (Pritchett et al. 2009).

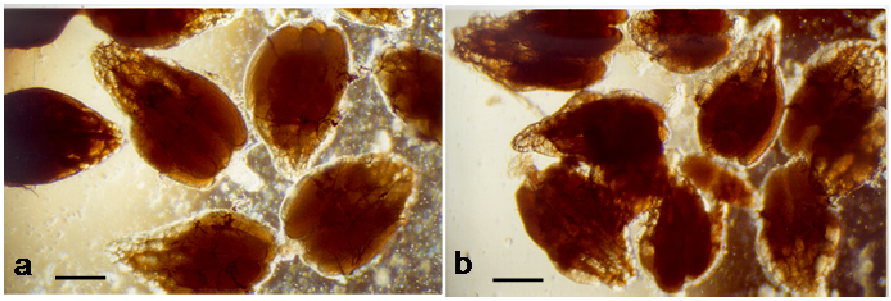

Figure 4. Effect of GSM 900 MHz radiation on ovarian development. a) Ovaries of sham-exposed virgin female flies 45 h after eclosion, b) Ovaries of exposed virgin female flies 45h after eclosion. The ovarian size of the exposed flies is significantly decreased in relation to the sham-exposed. Bars: 50 μm.

Stress-Induced DNA Damage and Cell Death during Oogenesis

Nurse cells and follicle cells, undergo programmed cell death (PCD) during the late developmental stages 11-14 of oogenesis, exhibiting chromatin condensation, DNA fragmentation and phagocytosis of the cellular remnants by the adjacent follicle and epithelial cells, events that are required for the normal maturation and ovulation of the egg chamber (Nezis et al. 2000; Pritchett et al. 2009; McCall 2004).

While cell death takes place physiologically in the nurse and follicle cells during late oogenesis, it rarely takes place physiologically during early and mid oogenesis and only at the two so-called “checkpoints”, germarium and stages 7-8 at the onset of vitellogenesis, after stress induction by environmental factors. Germarium (region 2) and the onset of vitellogenesis (stage 7-8) are called, early and mid-oogenesis “checkpoints” respectively, as they are the most sensitive developmental phases at which, abnormally developing follicles are eliminated by physiological apoptosis by the organism itself, in order to prevent the waste of

precious nutrients (Panagopoulos et al 2007a; Nezis et al 2000; Pritchett et al. 2009; McCall 2004).

Both check-points, are found to be very sensitive to stress factors like poor nutrition, (Drummond-Barbosa and Spradling 2001; Smith et al 2002), or exposure to cytotoxic chemicals like etoposide or staurosporine, (Nezis et al 2000). The mid-oogenesis checkpoint was at first observed, (Chao and Nagoshi 1999; De Lorenzo et al 1999; Nezis et al 2000), in response to cytotoxic chemicals and triggering the death of entire egg chambers during mid-oogenesis. Shortly after this the same checkpoint was found by other experimenters, (Drummond-Barbosa and Spradling 2001), to trigger cell death and egg elimination in response to poor nutrition stress. Additionally the same experimenters observed the other check point at the beginning of oogenesis, in the region 2a/2b of the germarium, to respond to poor nutrition stress.

Apart from these two check points, egg chambers were not observed before our experiments with mobile phone microwave exposure (Panagopoulos et al 2007a) to degenerate during other provitellogenic or vitellogenic stages, (from germarium up to stage 10), (Drummond-Barbosa and Spradling 2001; McCall 2004). In addition, before our experiments, induction of cell death was only observed in the nurse and follicle cells, and not in the oocyte (Nezis et al 2000; 2002; Drummond-Barbosa and Spradling 2001; McCall 2004)

A widely used method for identifying fragmented-damaged DNA is TUNEL assay. By use of this method, fluorescein dUTP is bound through the action of terminal transferase, onto fragmented genomic DNA which then becomes labelled by characteristic fluorescence. The label incorporated at the damaged sites of DNA is visualized by fluorescence microscopy (Gavrieli et al 1992). The application of TUNEL assay in *Drosophila* oogenesis is successfully used to demonstrate DNA fragmentation and cell death induction in regard to radiation, chemicals and other environmental factors (Panagopoulos et al 2007a; 2010; Nezis et al 2000; 2002; Drummond-Barbosa and Spradling 2001; Chao and Nagoshi 1999; De Lorenzo et al 1999; Giorgi and Deri 1976). For the application of the TUNEL assay in *Drosophila* oogenesis, see Panagopoulos et al 2007a, or Nezis et al 2000.

In our experiments with use of TUNEL assay, we have shown that microwave electromagnetic radiation emitted by mobile phones, can induce DNA damage and cell death in a significant percentage of egg-chambers during early and mid-oogenesis (up to 60%), only by a few min daily exposure during the first few days of the insects' adult lives, (Panagopoulos et al 2007a; 2010). In the same experiments we showed that the stress-induced cell death due to microwave radiation exposure, takes place not only at the two check-points, (germarium and

stages 7-8) but to all developmental stages of the early and mid-oogenesis, S1-S10, and even more at the germarium, suggesting that the germarium is an even more sensitive check-point than the mid-oogenesis one, at least in regard to electromagnetic stress. Moreover we showed that microwave exposure induced DNA damage not only in the nurse and follicle cells as other types of environmental stress, but also in the oocyte (Panagopoulos et al 2007a; 2010) (Fig. 5).

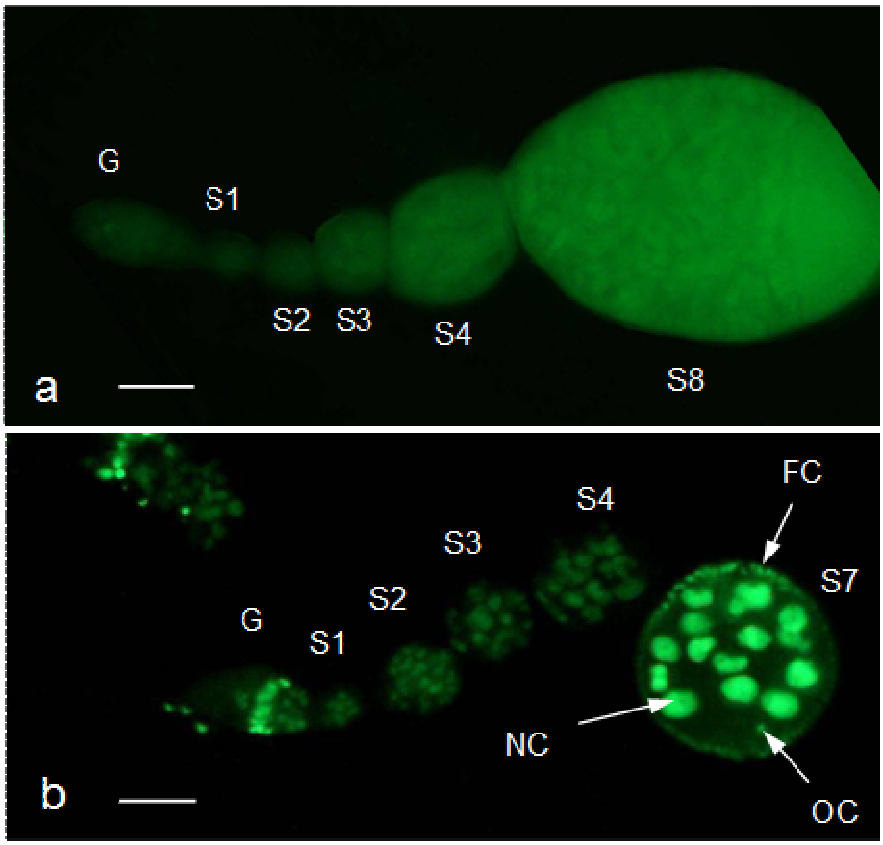

Figure 5. DNA damage in Drosophila egg cells after exposure to non-thermal microwave radiation as revealed by the TUNEL assay. a) Picture of ovariole of a sham-exposed insect with TUNEL-negative follicles, b) Ovariole of an exposed insect with TUNEL-positive signal denoting severe DNA damage, at all the developmental stages from germarium to stage 7 and in all three types of egg cells; nurse cells (NC), follicle cells (FC), and the oocyte (OC). Bars: 10 μm.

Thus, the significantly decreased oviposition and ovarian size recorded in our experiments with microwave exposure described above, are due to elimination of a significant percentage of egg-chambers during early and mid-oogenesis, which in turn is the result of cell death induction on the egg-chamber constituent cells.

In another series of experiments we showed by use of rhodamine-conjugated phalloidin staining assay alone or in combination with TUNEL assay, that the induced DNA damage is accompanied by, and coincides with, actin cytoskeleton damage, (Chavdoula et al 2010; Panagopoulos 2011a).

The decrease in the reproductive capacity caused by the alternating ELF magnetic field exposure was also found to be due to DNA damage induced by the magnetic filed. In contrast to the microwave exposure, and in spite of the longer exposure duration, (24 h daily for the five days) the magnetic field was found to induce DNA damage and consequent cell death only at the two check points (germarium and stages 7-8) and only in the nurse and follicle cells (not in the oocyte) just like with other types of environmental stress previously tested by other experimenters (starvation, chemicals). This possibly means that ELF magnetic fields although also genotoxic, represent a milder environmental stress factor than microwave radiation.

In the case of the pulsed electric field exposure, no statistically significant DNA damage or cell death induction was observed.

Conclusions

In the present chapter we presented experimental procedures for a fast and reliable assessment of environmental genotoxicity based on *Drosophila melanogaster* gametogenesis (especially oogenesis), reproduction, embryonic and post-embryonic development.

The above described experimental results of ours as well as of other experimenters show that microwave exposure even for a few min per day and for only a few days, at exposure levels encountered in our everyday environment, is maybe the most intense modern environmental stress factor compared to other environmental stress factors tested so far, like starvation, heat, chemicals, electric or magnetic fields. This derives from the result that the microwave radiation induced the largest decrease in reproductive capacity with the shortest exposure duration, and moreover from the fact that it induced DNA damage not only in the nurse and follicle cells and not only at the two check-points of oogenesis as other

environmental factors do, but also in the oocyte and at all the developmental stages of early and mid-oogenesis.

While DNA fragmentation in the nurse or follicle cells results in destruction and elimination of egg chambers, DNA damage in the oocyte which was found to take place only after microwave radiation exposure, may additionally result to inherited mutations transferred to subsequent generations. For this reason the biological changes due to microwave radiation may be far more dangerous as they may not be restricted only to changes in reproductive capacity.

As explained already, the oocyte seems to be much more resistant to all kinds of environmental stress, and also more protected than the other types of egg-chamber cells. This is possibly the reason why DNA damage was not observed to be induced by previously tested stress agents like chemicals or starvation in the oocyte, but it was only observed in the nurse and follicle cells.

The cell as a highly organized unit of life, has protective mechanisms against wrong cellular functions, e.g. by activating certain genes and consequently producing certain proteins like the "heat shock" ones, made to protect the cell from excessive heat. But if the cell fails to protect itself from an external disturbance, a malfunction may start which can be transferred to a whole organ or the whole organism. This is how biological effects become health effects. Electromagnetic fields seem to be perceived by the cells as external disturbances or external stress but the cells don't seem to have special genes to be activated for protection against electromagnetic stress. This seems to be the reason why in response to electromagnetic stress, cells activate heat shock genes and produce heat shock proteins very rapidly (within minutes) and at a much higher rate than for heat itself, (Weisbrot et al, 2003). It seems to be for the same reason that electromagnetic stress from microwave radiation used in modern mobile telecommunications, induces cell death in the reproductive cells much more than other types of external stress examined so far, like food deprivation, chemicals, heat etc.

In general, it seems that cells are much more sensitive to man-made electromagnetic stress than to other types of stress previously known. This is apparently due to the fact that this type of stress is new and possibly more intense, and cells have not developed defence mechanisms against it. DNA damage in somatic cells may also lead to mutations and cancer, if not to cell death. But apart from the DNA damage, the fact that cells activate heat shock genes to protect themselves from electromagnetic stress, and that this happens at a much higher rate than for heat itself, is also particularly dangerous, since repetitive stress leading to continuous expression of heat shock genes may also result to cancer induction (French et al, 2001).

Our above analysis shows that different modern environmental agents and in particular microwave and magnetic field exposure may induce DNA damage. This is shown in *Drosophila* reproductive cells, but according to our analysis, once this is shown in *Drosophila*, it may potentially be valid for other animal and human cells as well.

This possibility is supported by the experimental findings on reproduction decreases not only in *Drosophila* (Panagopoulos et al 2004; 2007a; 2007b; 2010) and other insects' population declines possibly related (Stindl and Stindl 2010; Bacandritsos et al 2010; van Engelsdorp et al 2008), but also by studies on birds (Everaert and Bauwens 2007; Balmori 2005), mammals (Magras and Xenos 1997; Gul et al 2009), and human reproductive cells as well (De Iouliis et al 2009; Agarwal et al 2009).

In addition, recent epidemiological findings tend to show brain cancer induction in mobile phone users (Khurana et al 2009; Hardell et al 2009; 2007). Brain cells are mainly nerve and glial cells. While glial brain cells may become cancerous after DNA damage leading to brain cancers, nerve brain cells do not divide and thus are not likely to become cancerous, (Mausset-Bonnefont et al 2004). DNA damage on nerve brain cells may then lead to cell death or malfunction which are both linked to neurodegenerative deseases such as Parkinson's and Alzheimer's. The incidence of such deseases is also increasing in recent years.

In the present chapter we attempted to show that *Drosophila melanogaster* is a unique tool for the study of biological and consequently health impacts of various stress agents encountered in our modern environment. In particular, the ovarian development of this experimental animal is most sensitive to environmental stress. The two out of three types of cells constituting the eggs (the nurse and follicle cells) of this animal are extremely sensitive to environmental stress, possibly due to the fact that, a) they are anyway programmed to die physiologically in support of the oocyte b) they are programmed to die prematurely and cause elimination of the whole egg whenever an environmental threat is detected able to damage oocyte at later developmental stages. This is a wisely developed mechanism to protect the future generations of the animal.

All of our complicated science could by no means develop such a wise and accurate protective mechanism as the one accounted for in *Drosophila* oogenesis, but it has come to the level to be able to detect and study such wisely developed protective mechanisms built by nature. While similar protective mechanisms certainly exist in all animals, *Drosophila*'s short life cycle and accordingly short biological cycles, plus the other advantages described, make this organism unique for a fast and accurate assessment of environmental threats.

Quick and reliable assessment of the biological activity of environmental threats and in particular radiation, is in our days an extremely important issue for public health. In the present chapter we showed that *Drosophila* reproduction is a unique tool for this.

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