Chris D. Geddes *Editor*

Microwave Effects on DNA and Proteins

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Preface

For many years, the scientific community has studied ionizing and nonionizing electromagnetic field (EMF) radiation, either stand-alone or in combination with other agents, with regard to possible adverse health effects on biological systems. In particular, the roles of microwaves have received much attention with significant discussion focused on the underlying mechanisms of the so-called *thermal* or *nonthermal* effects, i.e., the reaction-promoting effects that occur with microwaves, with notable attention also on the role of both microwave-generated reactive oxygen and nitrogen species. In addition, these adverse health effects can also be taken in light of the fact that microwaves are indeed used for biological sample preparation/destruction, such as in histopathology to reduce the preparation time of tissue sections for microscopy, or used in the microwave-based Lyse-it™ technology (www.lyse-it.com), which can rapidly lyse cells and fragment DNA (and RNA/proteins) into desired base pair sizes.

Given the increasing use of devices which continuously emit polarized fields, which are believed to be significantly more bioactive than natural unpolarized ones, I have subsequently invited a group of well-known internationally recognized scientists to contribute leading-edge articles on the roles and effects that microwaves have on biological systems, particularly with regard to their effects on DNA and proteins.

At this time, I would like to thank the contributors for their excellent and timely contributions to this notable volume.

Many thanks.

Baltimore, MD, USA
November 11, 2016

Chris D. Geddes

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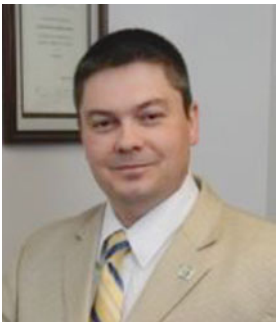
About the Authors

Dr. Jitendra Behari is Ph.D. in physics (1971) from the Indian Institute of Technology Delhi. He joined the Department of Biomedical Engineering of All India Institute of Medical Sciences, in 1973. Dr. Behari was professor in the School of Environmental Sciences of Jawaharlal Nehru University, New Delhi. He was conferred the honorary D.Sc. degree in bioelectromagnetics (Ansted University, UK). He has been a Fulbright fellow and adjunct faculty at Michigan State University, USA. He is a DAAD fellow and recipient of fellowship from the Japanese Association of Cultivation Sciences and British Council and has been a career awardee (University Grants Commission). He is a fellow of the Institution of Electronics and Telecommunication Engineers and the Ultrasonics Society of India and a senior member of the IEEE. He has been a member of Commission K of the URSI (engineering in medicine and biology society). Dr. Behari has been a past chairman of the IEEE, ED MTT Chapter. Presently, he is researching on the health implications of mobile phone frequency exposure.

Dr. Orlando Vieira Furtado-Filho graduated in biological sciences from the Educational Foundation of Alegrete, Brazil (Current University Campaign Region), and with a master's degree in biological sciences (molecular biology) from the University of Brasilia, Brazil (UNB), and a Ph.D. in biological sciences (cell and molecular biology) from the Federal University of Rio Grande do Sul, Brazil (UFRGS). He is major in Brazilian Army and a research collaborator of Biotechnology Center and Department of Biophysics, Institute of Biosciences, UFRGS. He has experience in molecular biology, biochemistry, and biophysics with emphasis on biochemistry of free radicals and metabolism of fatty acid, acting on the following topics: oxidants, antioxidants, oxidative damage to macromolecules, oxidative stress, free radicals, bioelectromagnetism, electromagnetic field, aging, and plant extract.

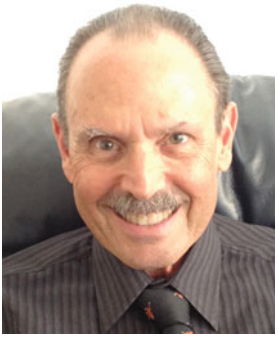


Dr. Chris D. Geddes, Ph.D., FRSC, and professor (www.chrisgeddes.com), has extensive experience in fluorescence spectroscopy, particularly in fluorescence sen sing, microwave-accelerated plasmonics, and metal-fluorophore interactions, publishing over 275 peer-reviewed papers (h-index: 43) and more than 30 books.

Dr. Geddes is internationally known in fluorescence and plasmonics, and his laboratory is widely attributed to the development of the metal-enhanced fluorescence (MEF) and related plasmon-fluorescence technologies, securing in excess of $25 million in recent years to pursue his research aspirations. He is the editor in chief of the *Journal of Fluorescence* and founding editor in chief of the *Who's Who in Fluorescence*, *Annual Reviews in Fluorescence*, and the *Annual Reviews in Plasmonics* volumes. In addition, due to the labs' pioneering efforts in the fields of metallic nanoparticle-fluorophore interactions, Dr. Geddes launched a Springer journal *Plasmonics* in 2005, which is a leading journal in the field today.

Dr. Geddes is director of the Institute of Fluorescence, a department within UMBC (University of Maryland, Baltimore County), which focuses on the nano-bio-technological applications of fluorescence. Dr. Geddes has been a permanent member of the NIH's EBIT R01 study section (2007–2012) and chaired the NIH's Analytical and BioAnalytical SBIR study section from ~ 2004 to 2009. Dr. Geddes is a fellow of both the Royal Society of Chemistry (FRSC) and the Institute of Physics. Dr. Geddes holds more than 100 patents in the fields of fluorescence and plasmonics, and his roles and interactions with industry have created enterprise value in excess of $100 million today. In 2011, Professor Geddes was honored by the Maryland House of Delegates in Annapolis, (House Resolution #326), for his outstanding contributions to education, biotechnology, economic development, and innovation.

Dr. Kazuhiko Imaizumi, Ph.D., D.V.M., received his bachelor of veterinary science from Tokyo University of Agriculture and Technology and Ph.D. in forensic medicine from Saitama Medical University. He is currently the chief of forensic anthropology laboratory in National Research Institute of Police Science, Japan. He has engaged in forensic identification of skeletal remains over 20 years and applied DNA identification technique to bone specimens at early stage of its development with studies on DNA extraction from compact bones. His current interest is three-dimensional morphological analyses in bone and face shapes that provide highly sophisticated scientific certainty in forensic anthropology. He is a fellow of the Japanese Association of Forensic Science and Technology.



Dr. Ronald N. Kostoff received a Ph.D. in aerospace and mechanical sciences from Princeton University in 1967. He has worked for Bell Laboratories, Department of Energy, Office of Naval Research, and MITRE Corp. He has published over 200 peer-reviewed articles, served as guest editor of four journal special issues since 1994, obtained two text mining system patents, and presently is a research affiliate at Georgia Institute of Technology. He has published on numerous medical topics in the peer-reviewed literature, including (1) potential treatments for multiple sclerosis, Parkinson's disease, Raynaud's phenomenon, cataracts, SARS, vitreous restoration, and chronic kidney disease, (2) potential causes of chronic kidney disease, and (3) potential impacts of electromagnetic fields on health.

Radiation biophysicist **Dr. Dimitris J. Panagopoulos** was born in Athens, Greece (December 6, 1961). He has a degree in physics and Ph.D. in biology from the University of Athens. He completed his Ph.D. on the biological effects of electromagnetic fields (EMFs) in 2001 and postdoctoral research on cell death induction by microwave radiation in 2006. Since 2002, he has been working at the section of cell biology and biophysics and has been giving lectures on radiation biophysics (physical properties and biological effects of ionizing and nonionizing radiations). Since 2014, he has been working at the National Center for Scientific Research "Demokritos," Laboratory of Health Physics, Radiobiology and Cytogenetics, researching effects of ionizing and nonionizing radiations on human cells. His experiments were among the first that showed effects of mobile telephony radiation on DNA, and his theory on the mechanism of action of EMFs on cells is considered the most valid.

Dr. Luc Verschaeve received Ph.D. in and is a professor of the University of Antwerp (faculty of biomedical sciences). He is responsible for the toxicology laboratory of the Scientific Institute of Public Health in Brussels and a present or past president and/or member of different national and international working groups on nonionizing radiations. Activities related to nonionizing radiation comprise research on the (genetic) toxicology of extreme low-frequency and radiofrequency fields (e.g., in the framework of the Belgian BioElectroMagnetics Group, cf. http://www.bbemg.ulg.ac.be/); the performing of many national projects involving the writing of information booklets and reports; communications for the general public, professionals, and authorities; etc. Research or involvements in activities related to nonionizing radiations started in 1992 and were since conducted without interruption. Another scientific research is mainly on natural products (e.g., mutagenicity and antimutagenicity of traditional medicinal plant extracts) (Scientific publications: more than 200 (from which more than 100 are referenced in PubMed)).

Dr. Takeo Yoshimura received his Dr. of Engineering degree from Kyushu Institute of Technology in 2009. He joined Kitakyushu Foundation for the Advancement of Industry Science and Technology as a researcher in 2009, and he transferred to Kyushu Institute of Technology as a research staff. From 2011 to 2014, he was an assistant professor of Tokyo University of Science. Since 2015, he joined Tokyo Institute of Technology as a researcher. Currently he joined SAIDA FDS INC., where he has been engaged in development of the microwave flow-reactor.

Chapter 1
Mobile Telephony EMFs Effects on Insect Ovarian Cells. The Necessity for Real Exposures Bioactivity Assessment. The Key Role of Polarization, and the "Ion Forced-Oscillation Mechanism"

Dimitris J. Panagopoulos

Abstract Exposure of *Drosophila melanogaster* young adult insects to Electromagnetic Fields (EMFs)/Radiation (EMR) emitted by an active GSM (Global System for Mobile telecommunications) mobile phone handset during a usual "talk" operation for a few minutes daily for 2–5 days, revealed an impressive decrease (up to 57%) in reproductive capacity (fecundity) (Panagopoulos et al. 2004). That effect directed us to focus our next studies on the effects of this type of EMF/EMR on the DNA and proteins of the insect's reproductive cells (gametes). More specifically, we focused on the effects on the female ovarian cells. We used the TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay, to detect fragmented DNA in the ovarian cells. Moreover, we used the Rhodamine-conjugated Phalloidin staining assay, to detect possible damage in the actin cytoskeleton of the ovarian cells. We found a high degree of DNA fragmentation in the nuclei of ovarian cells of the exposed insects (up to +55% compared to the sham-exposed insects) (Panagopoulos et al. 2007a). The DNA fragmentation was highly dependent on the intensity of radiation (distance from the handset) and was found to be maximum for intensities higher than 250 μW/cm^2 (in close proximity with the handset) and within a "window" around 10 μW/cm^2 (at 20–30 cm distance from the handset) (Panagopoulos et al. 2010). The DNA fragmentation in the nuclei of the exposed ovarian cells was found to be accompanied by actin cytoskeleton damage (Chavdoula et al. 2010). These effects caused a destruction of a significant percentage of egg chambers in the ovaries of the

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exposed females (Panagopoulos 2012a). New data (Panagopoulos et al. 2015a, b) suggest that the continuous and unpredictable variability of the mobile telephony signals, in combination with the fact that they are totally polarized (just like every type of man-made EMF), and the inclusion of Extremely Low Frequencies (ELF) - due to pulsing and modulation of the microwave carrier - in all modern mobile telecommunication microwave signals, constitute the main reasons for their intense bioactivity. A significant opposition is found between the results of experimental studies employing real exposures of biological samples from commercially available mobile phones, and the results of studies employing simulated exposures from generators or "test" phones as suggested by health authorities (Health Protection Agency 2012; IARC 2013). While experimental studies employing simulated EMF-emissions present a strong inconsistency among their results with nearly 50% of them reporting no effects, studies employing real-life emissions demonstrate an almost 100% consistency in showing adverse effects (Panagopoulos et al. 2015a). Finally, in the present chapter we show why polarized (man-made) EMFs are significantly more bioactive than natural (unpolarized) ones, and we describe the "Ion Forced-Oscillation Mechanism" for the action of polarized EMFs on biological systems.

Keywords Electromagnetic fields • Microwave radiation • Mobile phones • Real exposures • Biological effects • DNA damage • Protein damage • Reproduction • Ovarian cells • Insects • *Drosophila melanogaster* • Polarization • Mechanism of action

1.1 Introduction

Microwaves are called the electromagnetic waves produced by electronic oscillators of human technology, with frequencies higher than those which can be reflected by the ionosphere (thus higher than ~ 200 MHz) and up to the low limit of infrared (~ 300 GHz). Microwaves occupy the higher frequency part of a wider category called Radio-Frequency (RF) electromagnetic waves and start from frequencies around 10 kHz. In other words, microwaves are not reflected by the ionosphere unlike the electromagnetic waves of lower frequencies, and thus the receiving and the emitting microwave antennas need to have optical contact between them. The continuous demand for increasing the volume of transmitted information by microwave antennas leads to the continuous increase in the microwave frequencies, and the consequent approximation closer to the low limit of infrared (Lioliousis 1979).

In addition to the artificially produced microwaves which constitute the vast majority in our modern environment, there are natural microwaves of broad spectrum (10 MHz – 10 GHz) and cosmic origin which reach the earth's surface at very low intensities (~10^{-17} mW/cm^2/MHz) (Presman 1977). These are called cosmic microwaves. An important difference between man-made and natural (cosmic) microwaves is that the first are totally polarized like every form of man-made electromagnetic radiation (EMR)/field (EMF), while the second are unpolarized

just like every form of natural EMR/EMF. This difference is of decisive importance when it comes to the issue of the mechanism of how microwaves (and EMFs in general) interact with living matter, as we shall explain later on.

One of the main applications of man-made microwaves is in modern telecommunications, such as digital mobile telephony, domestic cordless phones (DECT-Digitally Enhanced Cordless Technology), or internet connection wireless devices and local wireless networks (Wi-Fi), or satellite communications. Radars employ microwaves as well.

With the tremendous development of telecommunications during the past few decades, the levels of RF/microwave radiation are exponentially increased especially in modern urban environments, and there is consequent increasing concern among the scientists and the public about the possible adverse health effects of these radiation types.

It is very important to emphasize that all (wireless) telecommunication devices emitting microwaves, emit extremely low frequencies (ELF) as well since this is absolutely necessary for pulsing and modulating the RF carrier signal in order to be able to transmit increasing amount of information (Tisal 1998; Hyland 2000; Hillebrand 2002; Tuor et al. 2005; Curwen and Whalley 2008). In other words, exposure to EMFs from mobile or cordless phones, Wi-Fi and other wireless communication devices, is simultaneous exposure to several frequencies both RF and ELF. It is suspected that the ELF emissions included in all telecommunication signals are those more responsible for the biological/health effects, and not the carrier RF signal itself (Panagopoulos et al. 2015b).

Hundreds of biological, clinical, and statistical/epidemiological studies investigating the potential health effects of microwave radiation are carried out so far. A first look shows contradicting results (Verschaeve 2009; Verschaeve et al. 2010; Bourthoumieu et al. 2010; Vijayalaxmi 2012; Health Protection Agency 2012; Ingole and Ghosh 2012; Ros-Llor et al. 2012; Karaca et al. 2012; Vignera et al. 2012; Waldmann et al. 2013; Cucurachi et al. 2013; IARC 2013; Balmori 2014; Paul et al. 2015; Lerchl et al. 2015; Roggeveen et al. 2015; Morgan et al. 2015; Singh et al. 2016; Manna and Ghosh 2016; Shahin-Jafari et al. 2016). But a closer look shows that there is a significant difference between studies employing simulated mobile phone signals from generators or "test" phones programmed to emit constant signals of fixed frequency, waveform and output power, and studies employing real signals from commercially available mobile phones or other microwave devices. While experimental studies employing simulated EMF-emissions present a strong inconsistency among their results with nearly 50% of them reporting no effects (Health Protection Agency 2012; Vijayalaxmi 2012; IARC 2013), studies employing real mobile phone exposures demonstrate an almost 100% consistency in showing adverse effects (Panagopoulos et al. 2015a). This consistency is in addition supported by studies showing association with brain tumors, symptoms of un-wellness, and declines in animal populations. These statistical/epidemiological studies concern exposures to real emissions as well, mostly from mobile phone handsets and base station antennas (Navarro et al. 2003; Salama and Abou El Naga 2004; Kundi 2004; Hutter et al. 2006; Balmori

2005, 2010; Balmori and Hallberg 2007; Everaert and Bauwens 2007; Khurana et al. 2009; Blettner et al. 2009; Kundi and Hutter 2009; Viel et al. 2009; Hardell et al. 2007, 2013; Bhattacharya and Roy 2014; Singh et al. 2016).

Determination of realistic exposures from mobile phones and other wireless telecommunication devices is one of the most important issues in scientific studies examining the biological/health effects of microwaves, since it is key to defining public health protection. The situation becomes confusing by the divergent results reported in the literature which can very well be due to unrealistic exposures, which in turn leads to wrong conclusions and ineffective and misdirected regulations (Panagopoulos et al. 2015a).

While the International Agency for Research on Cancer (IARC) has classified both ELF and RF EMFs as possibly carcinogenic to humans (IARC 2002, 2013), in its 2013 report IARC criticized and excluded from consideration experimental studies that used commercially available mobile phone handsets in exposing biological samples, as having "unreliable dosimetry" without further scientific explanation (IARC 2013). Similarly the Health Protection Agency criticized this exposure methodology reporting that the exposure is "highly variable" with "lack of control" due to network reasons (number of subscribers each moment) and movement of the animals within the vials/boxes in case of freely moving animals, but recognizes that restriction of the animals during the exposures will result in additional stress. Their critique recommended that exposures should be performed by devices or handsets set to produce emissions at fixed frequency and output power by use of engineering or hardware controls (Health Protection Agency 2012). In both reports the critique was based on the fact that real mobile phone emissions always include large variations in their intensity, frequency, etc., especially in the near-field of the antenna.

But billions of mobile phone users are daily exposed for increasing periods to real emissions from their handsets in the near-field of the antenna in contact with their ears/bodies, not to any simulated emissions with fixed parameters. Is it then scientifically correct to study the effects of a "highly variable" field by using fields with fixed parameters? In our opinion, it is definitely not. Especially since the varying nature of the field seems to be an important reason for its increased biological activity.

A plausible biophysical mechanism explaining how man-made EMFs can alter cell function by irregular gating of electrosensitive ion channels on the cell membranes is published (Panagopoulos et al. 2000a, 2002, 2015b) and verified by numerical test while previously suggested other mechanisms failed to pass the same test for realistic conditions within living cells (Halgamuge and Abbetrathne 2011). This mechanism known as "Ion Forced-Oscillation Mechanism" is based on the property of polarization of all man-made EMFs. Any externally applied polarized EMF will cause a parallel and in phase forced-oscillation of all charged particles - such as the mobile/free ions existing in large concentrations in all living cells - which can then exert constructive (additive) forces on any other charge such as the voltage-sensors of electrosensitive ion channels. These additive forces can be much more effective in gating (opening or closing) this type of ion channels than

the chaotic forces in every possible direction exerted by the same ions due to their random thermal motion, or due to any random individual oscillations caused by any unpolarized (natural) EMF.

In the present chapter we review a series of experiments with real mobile phone exposures that show important effects on DNA in insect ovarian cells. Then we examine the opposition between the results of studies employing real telecommunication microwave emissions and the results of studies employing exposures by simulated microwave emissions, as we think this is a point of utmost importance. Finally, we analyze the differences in bioactivity between polarized and unpolarized EMFs emphasizing on mobile telephony EMFs, and we provide an explanation of the reported biological effects on a biophysical basis according to the "Ion Forced-Oscillation Mechanism" for man-made (polarized) EMFs.

1.2 Effect of GSM Mobile Phone EMFs on Insect Ovarian Cells

1.2.1 Exposure Device and EMFs Measurements

We were the first to use a commercially available mobile phone handset to expose biological samples to microwave radiation (Panagopoulos et al. 2000b, 2004; Panagopoulos and Margaritis 2002). The reason was (and still is) obvious: We wanted to test the effects of the real EMFs which expose daily billions of humans globally.

In each study, in spite of the fact that we used a commercially available and therefore approved for the market mobile phone handset, we measured the intensity of radiation in the RF/microwave region during a normal phone-conversation. Moreover, we measured the electric and magnetic field intensities in the ELF region, since it is known that GSM emissions except for their carrier microwave frequency around 900, 1800 (Europe), or 1900 (North America) MHz, employ ELF pulsing fields at 217 Hz, and other even lower ELFs such as 8.34 Hz (Tisal 1998; Hyland 2000). The measurements and the experiments were always carried out with the battery of the handset fully charged, and at the same bench within the lab with full signal reception, and same positions of all items around (Panagopoulos et al. 2004, 2007a, b, 2010).

From the first sets of measurements we found out that when the mobile phone user talked during a phone conversation ("talk signal" or "voice-modulated signal" or "GSM basic") the intensities of the emissions increased about tenfold than when the user listened and there was no sound in the room ("listening mode" or "non-modulated signal" or discontinuous transmission mode-DTX). The biological effect of the voice-modulated exposures on the fecundity of young adult Drosophila insects was similarly much more intense than the corresponding effect of the DTX exposures with the same handset. While the DTX 900 MHz GSM exposures

Table 1.1 GSM 900 and 1800 radiation and field intensities ± SD, in the microwave and ELF bands averaged over 6 min of voice-modulated emission, for different distances from a mobile phone antenna[a]

Distance from mobile phone Antenna (cm)	**GSM 900** Radiation Intensity at 900 MHz, (mW/cm^2)	GSM 900 ELF Electric Field Intensity, (V/m)	GSM 900 ELF Magnetic Field Intensity, (mG)	**GSM 1800** Radiation Intensity at 1800 MHz, (mW/cm^2)	GSM 1800 ELF Electric Field Intensity, (V/m)	GSM 1800 ELF Magnetic Field Intensity, (mG)
0	0.378 ±0.059	19 ±2.5	0.9 ±0.15	0.252 ±0.050	13 ±2.1	0.6 ±0.08
1	0.262 ±0.046	12 ±1.7	0.7 ±0.13	0.065 ±0.015	6 ±0.8	0.4 ±0.07
10	0.062 ±0.020	7 ±0.8	0.3 ±0.05	0.029 ±0.005	2.7 ±0.5	0.2 ±0.05
20	0.032 ±0.008	2.8±0.4	0.2 ±0.04	0.011 ±0.003	0.6 ±0.12	0.1±0.02
30	0.010 ±0.002	0.7 ±0.09	0.1 ±0.02	0.007 ±0.001	0.3 ±0.06	0.06 ±0.01
40	0.006 ±0.001	0.2 ±0.03	0.05 ±0.01	0.004 ±0.0007	0.1 ±0.04	–
50	0.004 ±0.0006	0.1 ±0.02	–	0.002 ±0.0003	–	–
60	0.002 ±0.0003	–	–	0.0016 ±0.0002	–	–
70	0.0017 ±0.0002	–	–	0.0013 ±0.0002	–	–
80	0.0012 ±0.0002	–	–	0.0011 ±0.0002	–	–
90	0.0010 ±0.0001	–	–	0.0005 ±0.0001	–	–
100	0.0004 ±0.0001	–	–	0.0002 ±0.0001	–	–

[a]For distances longer than 30–50 cm from the mobile phone antenna, the ELF electric and magnetic field components of both GSM 900 and 1800 radiations, fall within the background of the stray 50 Hz fields within the lab

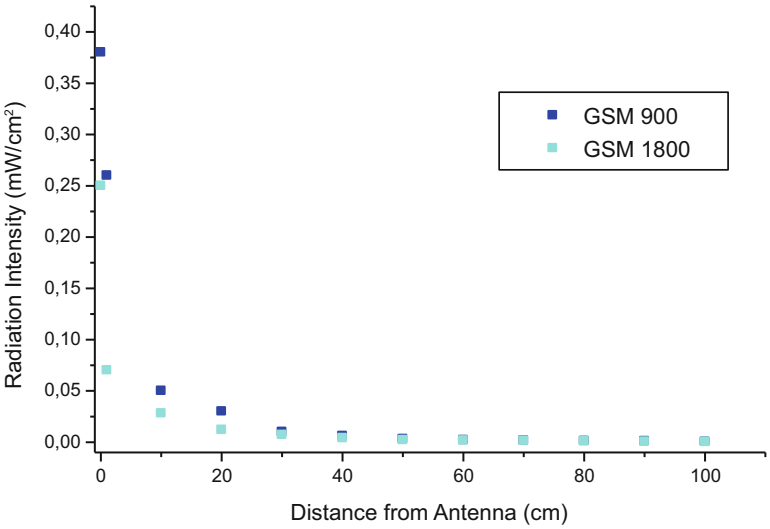

Fig. 1.1 Mobile phone radiation intensity values for GSM 900 and GSM 1800 MHz, according to the distance from the mobile phone antenna

of six min daily for 5 days reduced fecundity by approximately 18% compared to the unexposed insects, the voice-modulated ("talk") corresponding exposures reduced fecundity by approximately 53% respectively (Panagopoulos et al. 2004).

Representative intensity measurements of voice-modulated emissions averaged over 6 min, both in the microwave and ELF bands, and at different distances from the antenna of a mobile phone operating at 900 MHz and (the same handset with a different SIM - "subscriber identity module" card) at 1800 MHz, are shown in Table 1.1 (Panagopoulos et al. 2010). All mesured intensities are within the exposure criteria established by health authorities (ICNIRP 1998). The average radiation intensity levels of the microwave carrier (900 and 1800 MHz) according to the distance from the mobile phone antenna are graphically represented in Fig. 1.1. The standard deviations (SD) of the different intensity values given in Table 1.1 are not shown in Fig. 1.1. The ELF electric and magnetic field values also are given in Table 1.1 but not represented in the diagram as they are more or less proportional to the radiation intensity values.

1.2.2 Experimental Animals

Our experimental animals were *Drosophila melanogaster* flies, strain Oregon R wild-type, held in glass bottles with their food and kept in an incubator at 25 °C, with 12-h periods of light and darkness, and 70–75% relative humidity slightly varying from one experimental series to another but kept constant during each specific

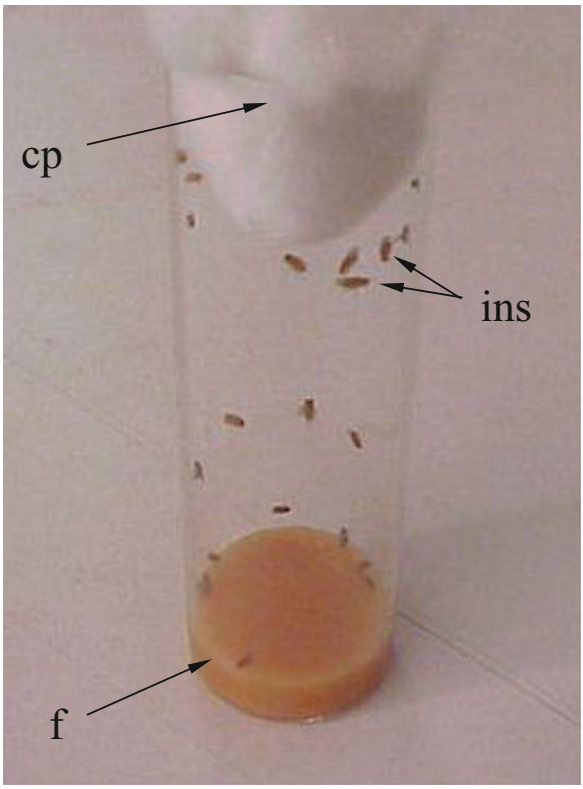

Fig. 1.2 A typical glass vial used in our experiments, containing a group of 20 insects (ins) and food (f), closed with cotton plug (cp)

experimental series. In each individual experiment we collected newly emerged adult flies which we separated into different groups of usually ten males and ten females (except when otherwise mentioned), and put within identical 50-ml cylindrical glass vials with 2.5 cm diameter and 10 cm height, with equal amounts of food of identical quality prepared at the same time, as previously described (Panagopoulos et al. 2004). Figure 1.2 shows one group of flies within a 50-ml glass vial with food.

The oogenesis of this insect is a model biological system, with a very good timing of developmental processes under controlled conditions (King 1970; Panagopoulos 2012b).

The reproductive capacity was assessed by the number of first filial generation (F_1) pupae derived from eggs laid during the first 3 days of the insect's maximum fecundity (oviposition). This number - under the conditions of our experiments - equals the number of fertilized laid eggs, since there is no statistically significant mortality of fertilized eggs, larvae or pupae derived from newly eclosed adult flies during the first days of their maximum oviposition (Panagopoulos et al. 2004, 2007a, b, 2010, 2013a; Panagopoulos 2012b).

1.2.3 Exposure Procedure

In each experiment, the collected newly emerged flies from the stock were anesthetised very lightly with diethyl ether and separated males from females. We put the collected flies in groups of usually ten males and ten females (twenty flies in each group) in the 50-ml vials with food, closed with cotton plugs.

In each experiment, the males and the females of each group were kept and exposed/sham-exposed in separate vials during the first 48 h (two separate vials for each exposed/sham-exposed group). Newly emerged adult *Drosophila* flies are not sexually mature immediately after eclosion. Male flies become sexually mature about 12 h after eclosion and females about 45 h after eclosion (King 1970; Panagopoulos 2012b). Keeping males separately from females for the first 48 h of each experiment ensures that when the two sexes are put together they are in complete sexual maturity and ready for immediate mating and laying of fertilized eggs. This in turn ensures that all laid eggs during the next 72 h are fertilized, and this minimizes variability in oviposition counts.

After the first 48 h, the males and females of each group (20 flies) were lightly anesthetized again and put together in a single vial with fresh food (one vial for each group) to continue being daily exposed/sham-exposed and allowed to mate and lay eggs for the next 72 h, during which the insect's oviposition is at its maximum (King 1970; Bos and Boerema 1981; Panagopoulos et al. 2004; Panagopoulos 2012b).

The exposure to the GSM fields started from the first day of the experiments, 1 h after the insects were fully awaken from the first anesthesia and - in most experiments - lasted for 6 min daily in a single exposure. In experiments testing the effect of exposure duration the daily exposures ranged from 1 to 21 min between the different groups (Panagopoulos and Margaritis 2010a). The exposures took place for the first 2–6 days of the insects' adult lives, depending on the experimental protocol. In experiments comparing the effect of the EMF on the reproductive capacity between the two sexes the exposures took place only for the first 2 days (while the two sexes in all groups were separated) (Panagopoulos et al. 2004). In experiments testing the effect on reproductive capacity of both sexes the EMF exposures took place for the first 5 days of the insects' adult lives (Panagopoulos et al. 2004, 2007b; Panagopoulos and Margaritis 2010a, b). In experiments testing the effect on DNA and proteins, there was an additional exposure in the sixth day before dissection and treatment of the ovaries (Panagopoulos et al. 2007a, 2010; Chavdoula et al. 2010).

In the different sets of experiments we separated the insects into: a) the Exposed group(s) and b) the Sham-Exposed group(s). The sham-exposed groups had identical treatment with the exposed groups, except that the mobile phone handset was turned off during the sham-exposures. We performed several replicate experiments for each different experimental protocol in the different studies.

The number of groups depended on the specific experimental protocol. For example, in the first experiments testing the effect of GSM radiation on

reproduction (fecundity) we separated the insects into two groups (one exposed and one sham-exposed) (Panagopoulos et al. 2000b, 2004; Panagopoulos and Margaritis 2002). In the set of experiments testing the dependence of fecundity/DNA damage on the intensity of the radiation/fields, we exposed simultaneously 12 groups at 0, 1, 10, 20,. . ., 100 cm distance from the mobile phone, and thus we had 12 Exposed groups plus one sham-exposed (13 groups in each replicate experiment) (Panagopoulos et al. 2010). In the set of experiments testing the effect of exposure duration on reproductive capacity (Panagopoulos and Margaritis 2010a), we separated the insects into six groups: (a) a group exposed to the GSM EMFs for 1 min, (b) a group exposed for 6 min, (c) a group exposed for 11 min, (d) a group exposed for 16 min, (e) a group exposed for 21 min, and (f) the sham-exposed group (SE). [Details for each study can be found in the original publications].

The temperature in the laboratory during the experimental procedures was maintained at 24 ± 0.5 °C. No detectable temperature increase took place within the vials with the insects or within the mass of the food due to the exposures at all distances between the handset and the vials, and with all tested exposure durations. That was checked during preliminary exposures in each study by the use of a Hg-thermometer with 0.05 °C sensitivity.

1.2.4 Reproductive Capacity Assessment

When the male and female flies of each group had been together in the same vial for 3 days (72 h), that is after 5 days from the beginning of each experiment, the flies were removed from the glass vials. These vials containing the developing embryos were then kept in the culture room for at least six additional days without any further EMF exposure/sham-exposure.

After the six additional days that the vials without the parental flies were kept in the culture room without exposure, most F_1 embryos (deriving from the fertilized laid eggs) were at the stage of pupation, where they could be clearly seen macroscopically and easily counted on the walls of the glass vials. [At the last larvae stages the larvae go out of the food, crawl up on the walls of the glass vials and get immobilized there to become pupae (Panagopoulos 2012b)].

By counting the F_1 pupae 11 days after the beginning of each experiment (2 days separated males from females, plus 3 days mating and egg laying, plus 6 days for the F_1 embryos to reach the pupation stage), we had a representative estimate of each group's reproductive capacity. In this way, instead of counting eggs under a stereo microscope on the surface and within the food which is subject to large errors, we simply counted the number of pupae with bare eyes on the walls of the glass tubes with no error at all. That was also an important innovation in assessing the fecundity of this insect, in addition to keeping the males and females of each group in separate vials for the first 48 h of each experiment (Panagopoulos et al. 2004). All procedures and counts were performed blindly (the experimenter did not know the identity of the groups).

The blinded scoring was repeated for the next 2 days in order to ensure that no larvae had remained within the food and all F_1 embryos had reached the pupation stage and were counted. In this way, no error can occur in the scoring of F_1 pupae.

Comparing the numbers of F_1 pupae between exposed and sham-exposed groups, we get a credible estimate of the effect of EMF exposure on reproductive capacity.

1.2.5 Assessment of DNA Fragmentation in the Ovarian Cells

Oogenesis in *Drosophila* starts during the last stages of pupation. At eclosion, the ovaries of female flies contain already eggs at the first preyolk stages. The eggs develop through 14 distinct stages until they are ready to be fertilized and laid. This process for every egg lasts about 48 h at 25 °C (King 1970; Panagopoulos 2012b).

In order to investigate the ability of the EMFs to damage DNA during early and mid oogenesis (when programmed cell death does not occur) we applied the TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay which is a marker for DNA fragmentation. In this assay fluorescein dUTP (a fluorescent substance) binds through the action of terminal transferase (an enzyme that catalyzes the specific biochemical reaction), onto broken chains (phosphodiester bonds) of genomic DNA which then become labelled by this fluorescent substance. The label incorporated at the damaged sites of DNA is visualized by fluorescence microscopy, as it emits characteristic visible radiation when it is irradiated by ultraviolet ("TUNEL-positive signal").

In the studies testing the effect of EMFs on ovarian DNA and proteins there was an additional exposure in the morning of the sixth day of each individual experiment as already reported. After 1 h from this additional exposure, the parental flies were removed from the glass vials, and they were anesthetized and sacrificed. The ovaries from all exposed and sham-exposed female flies were dissected in Ringer's solution and either were collected intact to measure the ovarian size (Panagopoulos 2012a), or separated into individual ovarioles and egg chambers from which the egg chambers of stages 11–14 were excluded (Panagopoulos et al. 2007a, 2010, 2013a; Chavdoula et al. 2010; Panagopoulos 2016). In egg chambers of stages 11–14 programmed cell death takes place physiologically in the nurse and follicle cells (McCall 2004; Nezis et al. 2000, 2002). Thereby we kept and treated ovarioles and individual egg chambers from germarium up to stage 10. A few representative samples were selected from both ovaries of all the exposed and sham-exposed females in each experiment. The selected samples of ovarioles and egg chambers were then fixed for the TUNEL assay or the rhodamine-conjugated phalloidin staining assay, to search for DNA damage or actin cytoskeleton damage (Panagopoulos et al. 2007a, 2010, 2013a; Chavdoula et al. 2010; Panagopoulos 2012a, 2016).

The application of the TUNEL assay is described in detail in Panagopoulos et al. (2007a). The samples were viewed under a Nikon EZ-C1 fluorescence microscope (Nikon Instruments, Japan). Samples from different groups were blindly observed under the fluorescence microscope (i.e. the observer did not know the origin of the sample) and the percentage of egg chambers with TUNEL-positive signal was scored in each sample.

1.2.6 Assessment of Actin Cytoskeleton Damage in the Ovarian Cells

In this set of experiments - a detailed description can be found in Chavdoula et al. (2010) - we examined whether the GSM exposure induces disorganization of the actin cytoskeleton in the reproductive cells of exposed female insects during early and mid oogenesis. The disorganization of the actin cytoskeleton is a known aspect of cellular death during both apoptosis and necrosis. For this we applied the rhodamine-conjugated phalloidin staining assay. Rhoramine is a fluorescent substance that gets attached to the actin cytoskeleton through the binding of phalloidin. When this is done, the morphology of the actin cytoskeleton is observed by fluorescence microscopy.

We also examined whether follicles with TUNEL-positive signal in their constituent cells had at the same time alterations in their actin cytoskeleton. For this we used double staining with rhodamine-conjugated phalloidin and TUNEL assay at the same samples. The simultaneous observation of DNA fragmentation and actin cytoskeleton damage was accomplished by double action of two different lasers on the samples and observation of the corresponding two types of fluorescence through a Nikon EZ-C1 Confocal Laser Scanning Microscope (Nikon Instruments, Japan) (Chavdoula et al. 2010).

1.2.7 Results

1.2.7.1 GSM EMFs Dramatically Decrease Reproduction

The results of the experiments investigating the effect on reproduction showed that the 6 min daily exposure to GSM EMFs in “talk” mode at close proximity (near-field) to the mobile phone antenna during the first 5 days of the insects’ adult lives, induced an impressive average decrease by 53.01% in their reproductive capacity (fecundity). The corresponding average decrease in DTX mode was 18.24%. The GSM field was found to decrease the reproductive capacity in both male and female insects (Panagopoulos et al. 2004).

1.2.7.2 The Decrease in Reproduction Is Due to Elimination of Egg Chambers After DNA Fragmentation and Consequent Cell Death of Their Constituent Cells

The dramatic decrease in reproductive capacity was found to be due to destruction of significant numbers of egg chambers after severe DNA damage in the reproductive cells (gametes). This was found both for GSM 900 and GSM 1800 radiation types. The effect with GSM 900 was more intense than the corresponding effect with GSM 1800 mainly due to its higher intensity, and in a much lesser degree due to its lower carrier frequency (Panagopoulos et al. 2007a, b).

Figure 1.3 shows an ovariole of a sham-exposed female insect with no DNA fragmentation at all developmental stages. This TUNEL-negative picture was representative of the ovarioles of all sham-exposed insects. Figure 1.4 shows an ovariole of an exposed insect with DNA fragmentation (TUNEL-positive signal) only at the two most sensitive developmental stages/checkpoints (germarium and stage 7–8), and no DNA fragmentation (TUNEL-negtive signal) at all other developmental stages. Figure 1.5 shows ovarioles of exposed insects with DNA fragmentation (TUNEL-positive signal) at all developmental stages from germarium up to stage 8. Figure 1.6 shows a stage 10 egg chamber with DNA fragmentation (TUNEL-positive signal) in the nurse and follicle cells.

What was novel and impressive with these findings was that the GSM EMFs induced DNA fragmentation, (1) at all developmental stages of early and mid oogenesis while previously examined stress factors such as starvation or chemicals were found to induce DNA damage only at the two most sensitive developmental stages/checkpoints (germarium and stages 7–8). (2) The DNA fragmentation was observed in all three types of egg chamber cells (nurse cells, follicle cells, and the oocyte), while the above previously examined stress factors induced DNA damage only in the nurse and follicle cells, not in the oocyte (Nezis et al. 2000, 2002; Drummond-Barbosa and Spradling 2001; Panagopoulos et al. 2007a, 2010).

1.2.7.3 The DNA Damage in the Gametes and the Consequent Decrease in Reproduction Is Primarily Dependent on the Intensity of the GSM Fields

The effects on reproduction and on DNA damage decreased non-linearly with increasing distance from the mobile phone handset, or with decreasing EMFs intensities. The effects were maximum for radiation intensities higher than 250 $\mu W/cm^2$ (close proximity with the mobile phone antenna) and within a "window" around 10 $\mu W/cm^2$ (20–30 cm from the handset) (Panagopoulos et al. 2010). More specific experiments showed that the discovered "window" of maximum bioactivity was not dependent on any specific position but on the specific of EMF-intensity values (Panagopoulos and Margaritis 2010b).

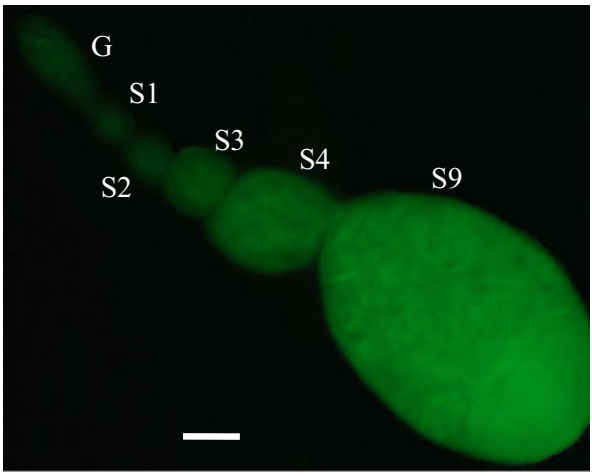

Fig. 1.3 Typical TUNEL-negative fluorescent picture of an ovariole of a sham-exposed female insect, containing egg chambers from germarium to stage 9. Bar: 10 μm

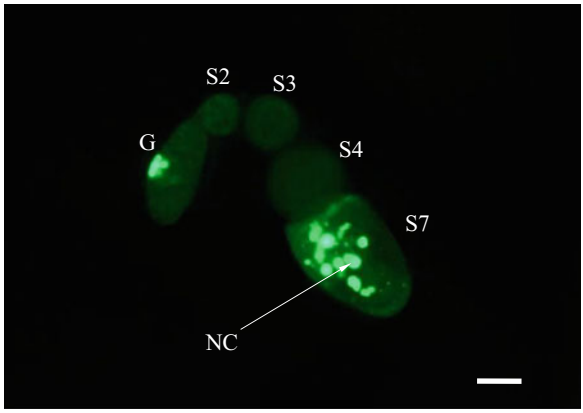

Fig. 1.4 Ovariole of an exposed female insect with TUNEL-positive signal in the nurse cells (NC) only at the two check points, germarium (G) and stage 7 egg chamber, and TUNEL-negative intermediate stages. Bar: 10 μm

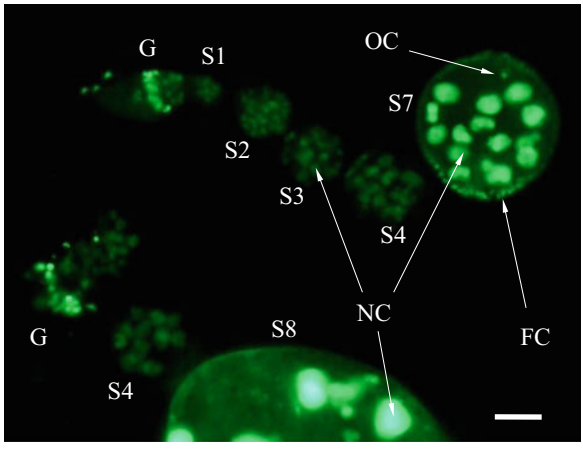

Fig. 1.5 Ovarioles of an exposed female insect with fragmented DNA at all developmental stages from germarium (G) to stage 8, and in all kinds of egg chamber cells, (NC: nurse cells, FC: follicle cells, OC: oocyte). Bar: 10 μm

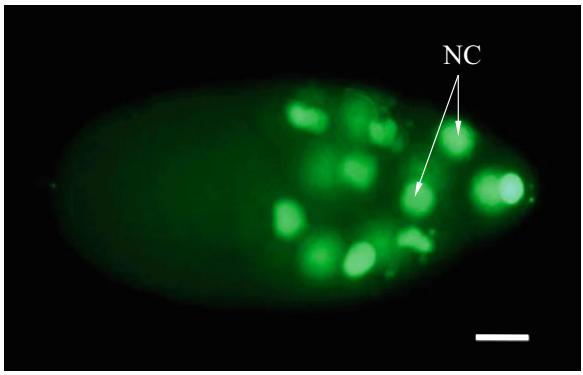

Fig. 1.6 A stage 10 egg chamber of an exposed female insect with fragmented DNA in the nurse cells, (NC). Bar: 10 μm

Figures 1.7 and 1.8, show the percentage of egg chambers with fragmented DNA and the reproductive capacity, in accordance to the distance of the vials from the mobile phone handset for GSM 900 and 1800 respectively. The effects manifested for intensities down to 1 $\mu W/cm^2$ corresponding to approximately 1 m distance from the handset (Panagopoulos et al. 2010).

The carrier frequency of the GSM radiation (900 or 1800 MHz) was found to have only a minimal effect on ovarian DNA fragmentation and the reproductive capacity, with 900 MHz being slightly more bioactive than 1800 MHz under the same EMFs intensities (Panagopoulos et al. 2007a, b, 2010). For example with radiation intensity approximately 285 $\mu W/cm^2$, GSM 900 decreased fecundity by 32.75% in relation to the sham-exposed group while GSM 1800 decreased fecundity by 31.08% (Panagopoulos et al. 2007b). [The "Ion Forced-Oscillation Mechanism" described below shows that the bioactivity is inversely proportional to the frequency of the EMF (Eq. 1.24)].

1.2.7.4 The Effect on Reproduction Increased with Increasing Daily Exposure Durations

In this set of experiments the insects were exposed at the far field of the antenna where previous experiments (Panagopoulos et al. 2010) showed that we have the largest effect, that is at 30 cm distance from the handset for GSM 900 or at 20 cm distance for GSM 1800 where the intensity of both radiation types was ~10 $\mu W/cm^2$. The groups were exposed simultaneously, placed along constant intensity sectors of an arc with a 30- or 20-cm radius - for GSM 900 or GSM 1800 respectively - at the center of which the handset was placed. Then, during each exposure session, the different groups were taken away from the exposure bench one by one, as soon as the exposure duration of each one was completed. (Panagopoulos and Margaritis 2010a).

The experiments showed that even 1 min of daily exposure during the first 5 days of the insects' adult lives is capable to induce a significant decrease in reproduction on the order of 35% in relation to the sham-exposed insects. Then, as the duration of

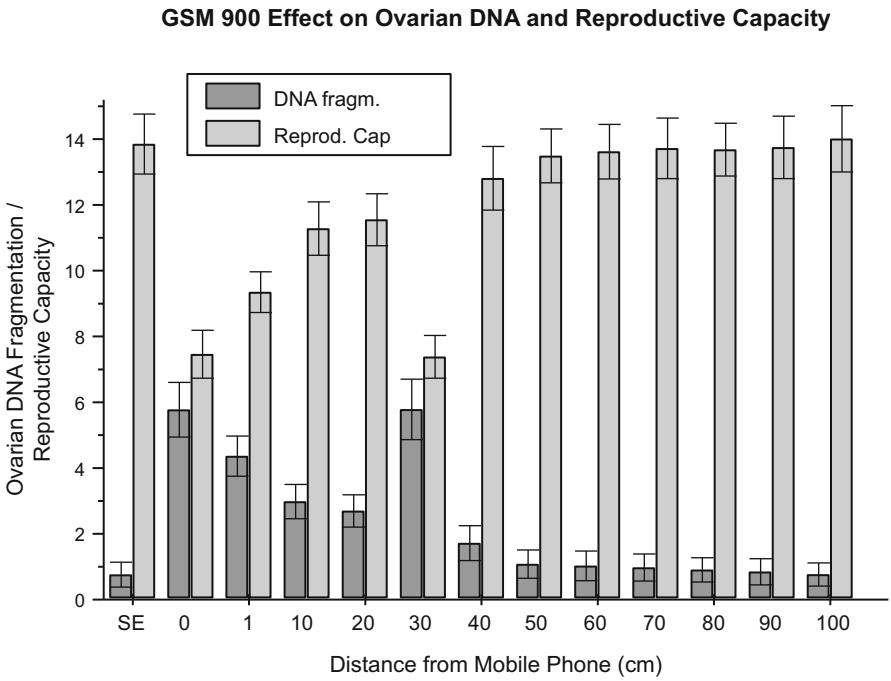

Fig. 1.7 [Mean ratio of ovarian DNA fragmentation (number of TUNEL- positive to total number of egg-chambers) ± SD]×10, and Mean number of F_1 pupae per maternal insect ± SD, versus Distance from mobile phone antenna (cm), for GSM 900

the single daily GSM exposure gradually increased from 1 to 21 min, fecundity was decreasing almost linearly. The effect was shown both with GSM 900 or GSM 1800 fields, but again, GSM 900 was found to be slightly more bioactive than GSM 1800 (Panagopoulos and Margaritis 2010a).

Figure 1.9 shows the decrease in reproduction in relation to the exposure duration for GSM 900, and 1800.

1.2.7.5 The DNA Damage in the Exposed Ovarian Cells Was Found to be Accompanied by Actin Cytoskeleton Damage

The same cells that suffered DNA fragmentation after exposure to the GSM fields, also suffered actin cytoskeleton disorganization as it was shown by double staining of the ovarian samples with both TUNEL and rhodamine-conjugated phalloidin, and observation by two different lasers under a Confocal Laser Scanning Microscope. The percentages of egg chambers with actin cytoskeleton damage in both exposed and sham-exposed samples were very close to corresponding percentages of DNA damage. The analysis by double-staining with TUNEL and rhodamine conjugated phalloidin revealed that DNA fragmentation and actin-cytoskeleton

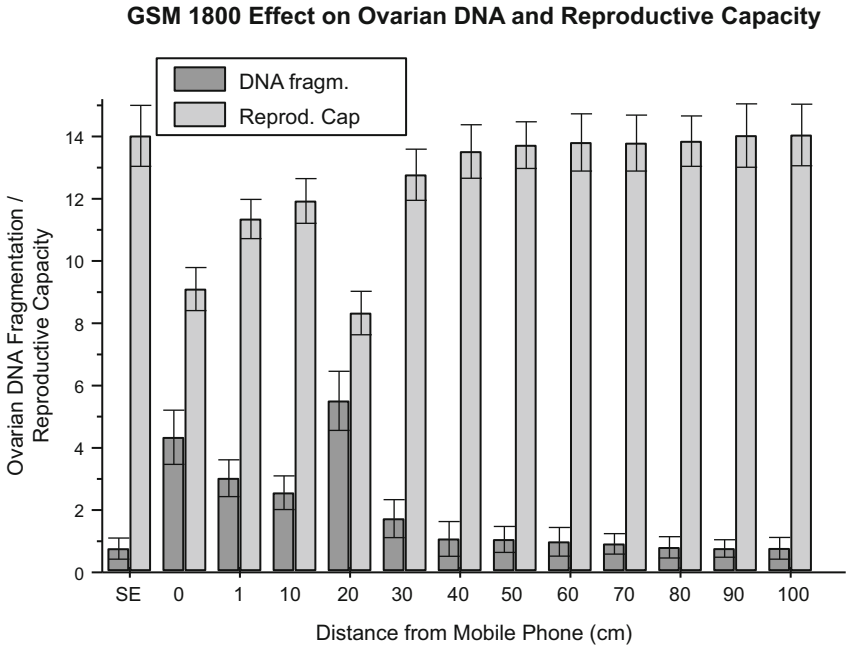

Fig. 1.8 [Mean ratio of ovarian DNA fragmentation (number of TUNEL- positive to total number of egg-chambers) ± SD]×10, and Mean number of F_1 pupae per maternal insect ± SD, versus Distance from mobile phone antenna (cm) for GSM 1800

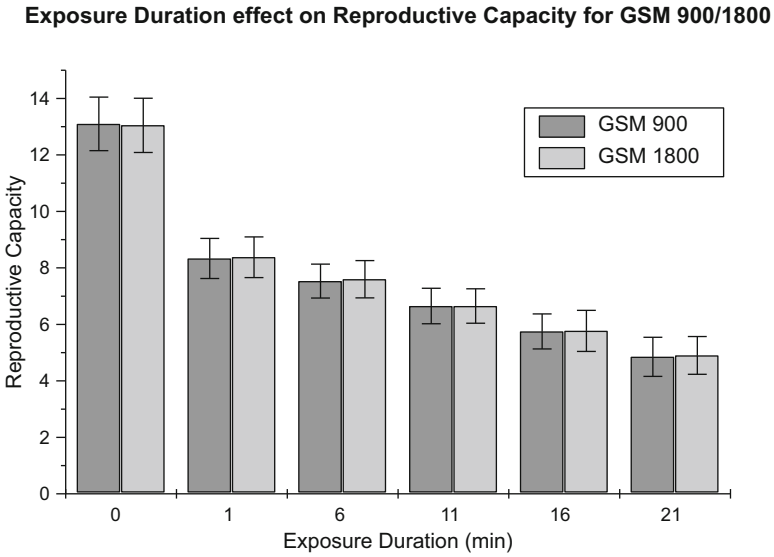

Fig. 1.9 Reproductive capacity ± SD of groups exposed to GSM 900 or 1800 fields for different daily exposure durations (1, 6, 11, 16, and 21 min) and of sham-exposed groups (0 min)

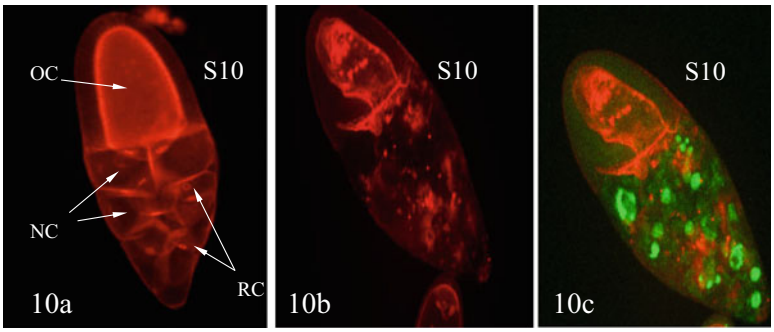

Fig. 1.10 (**a**) A stage 10 egg chamber from a sham exposed insect, treated with rhodamine-conjugated phalloidin assay, with normal cytoskeleton morphology. Characteristic features of the actin cytoskeleton like the ring channels (RC) can be observed. *NC* nurse cells, *OC* oocyte. (**b**) A stage 10 egg chamber of an exposed insect with disorganized actin cytoskeleton. (**c**) The same stage 10 egg chamber as in (**b**), treated with both TUNEL (*green* fluorescence) and rhodamine-conjugated phalloidin (*orange* fluorescence) assays, revealing that DNA fragmentation and actin cytoskeleton disorganization coexist in the damaged follicles of the exposed insects

disorganization coincide in the affected egg chambers. Since the actin cytoskeleton damage is a known sign of cell death, this result shows that the affected cells most usually do not survive, and are led to apoptosis (Chavdoula et al. 2010).

Figure 1.10a shows the actin cytoskeleton of a stage 10 egg chamber of a sham-exposed insect with normal morphology. Specific features such as the ring channels can be clearly seen. Figure 1.10b shows a stage 10 egg chamber of an exposed insect with damaged actin cytoskeleton. Figure 1.10c shows that the actin cytoskeleton damage accompanies the DNA damage in the same cells (Chavdoula et al. 2010).

1.2.7.6 The Ovarian Development in the Exposed Females Was Significantly Decreased

Due to the DNA and actin cytoskeleton damage induced in the egg chamber cells by the GSM EMFs and the consequent cell death in the affected cells, a large percentage of the developing egg chambers in the ovaries of exposed females were eliminated. As a result, the ovarian size of the exposed females was significantly decreased compared to the ovarian size of the unexposed insects. This we showed with just eclosed virgin female adult insects which we exposed for 6 min 3 h after eclosion, and subsequently every 10 h. Then the ovaries of exposed and sham-exposed insects were dissected and photographed under the same magnification at different developmental stages during the first 2 days (48 h) of their adult lives which is a little more than the time needed for the complete development of the first egg chambers in the ovaries under certain controlled laboratory conditions (~ 45 h). The average ovarian size was compared between exposed and sham-exposed insects according to the photographs. Figure 1.11a, b show ovaries of

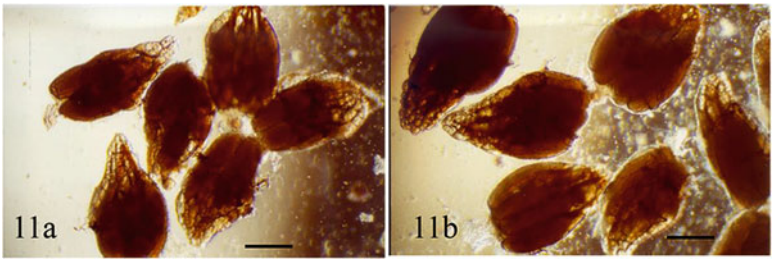

Fig. 1.11 Ovaries of exposed (**a**) and sham-exposed (**b**) female insects 45 h after eclosion. Ovaries of exposed insects are significantly smaller than those of sham-exposed, due to elimination of egg chambers after cell death of their constituent cells induced by the GSM field. Bars: 50 μm

exposed and sham-exposed insects respectively, 45 h after eclosion. The average "descriptive ovarian size" (DOS) of the exposed ovaries was decreased by 29.75% compared to the sham-exposed (Panagopoulos 2012a).

1.2.7.7 Microwave GSM Field Was Found to be Considerably More Bioactive than ELF Magnetic Field or Pulsed Electric Field. The Differential Bioactivity Between Different Types of EMFs Revealed a Differential Sensitivity Between Different Types of Cells, and Between the Two Checkpoints of Oogenesis

The exposure to the GSM field induced more DNA damage on the same biological system than exposure to 50 Hz alternating magnetic field with intensities 1–21 G, or 8 kHz electric field pulsed on 44.4 Hz with intensities 100–400 kV/m, even though the exposures to these fields were of significantly longer daily exposure durations, and of intensities exceeding the environmentally accounted ones (Panagopoulos et al. 2007a, 2013a; Panagopoulos 2016). While the GSM 6-min exposures for a few days induced DNA damage in the ovarian cells up to +55% compared to the sham-exposed cells, the 50 Hz alternating magnetic field induced DNA damage up to +7.52% with the strongest intensity (21 G), and the pulsed electric field up to +3.87% with the strongest intensity (400 kV/m).

This differential bioactivity of the different types of EMFs, revealed that there is a differential sensitivity between the three types of egg chamber cells (NC-nurse cells, FC-follicle cells, OC-oocyte). It was found that cellular sensitivity to EMFs decreases (or resistivity increases) from the NC to the FC, and from the FC to the OC. In other words it was found that the OC (the single cell in each egg chamber that will give the offspring after fertilization) is the most resistant cell type, and the NC the most vulnerable from the three cell types to EMF-exposure (Panagopoulos 2007a, 2013a; Panagopoulos 2016).

Moreover, it was found that the two checkpoints of oogenesis (germarium, and stage 7–8) present different sensitivities in regards to exposures to different types of