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ORIGINAL PAPER

ELF Alternating Magnetic Field Decreases Reproduction by DNA Damage Induction

Dimitris J. Panagopoulos · Andreas Karabarbounis · Constantinos Liolioussis

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Abstract In the present experiments, the effect of 50-Hz alternating magnetic field on *Drosophila melanogaster* reproduction was studied. Newly eclosed insects were separated into identical groups of ten males and ten females and exposed to three different intensities of the ELF magnetic field (1, 11, and 21 G) continuously during the first 5 days of their adult lives. The reproductive capacity was assessed by the number of F_1 pupae according to a well-defined protocol of ours. The magnetic field was found to decrease reproduction by up to 4.3 %. The effect increased with increasing field intensities. The decline in reproductive capacity was found to be due to severe DNA damage (DNA fragmentation) and consequent cell death induction in the reproductive cells as determined by the TUNEL assay applied during early and mid-oogenesis (from germarium to stage 10) where physiological apoptosis does not occur. The increase in DNA damage was more significant than the corresponding decrease in reproductive capacity (up to $\sim$7.5 %). The TUNEL-positive signal denoting DNA fragmentation was observed exclusively at the two most sensitive developmental stages of oogenesis: the early and mid-oogenesis checkpoints (i.e. region 2a/2b of the germarium and stages 7–8 just before the onset of vitellogenesis)—in contrast to exposure to microwave radiation of earlier work of ours in which the DNA fragmentation was induced at all developmental stages of early and mid-oogenesis. Moreover, the TUNEL-positive signal was observed in all three types of egg chamber cells, mainly in the nurse and follicle cells and also in the oocyte, in agreement with the microwave exposure of our earlier works. According to previous reports, cell death induction in the oocyte was observed only in the case of microwave exposure and not after exposure to other stress factors as toxic chemicals or food deprivation. Now it is also observed for the first time after ELF magnetic field exposure. Finally, in contrast to microwave exposure of previous experiments of ours in which the germarium checkpoint was found to be more sensitive than stage 7–8, in the magnetic field exposure of the present experiments the mid-oogenesis checkpoint was found to be more sensitive than the germarium.

Keywords Electromagnetic fields (EMFs) · Alternating magnetic field · Extremely low frequency (ELF) · Biological effects · Drosophila melanogaster · DNA damage

D. J. Panagopoulos (✉)
Department of Biology, University of Athens,
Panepistimiopolis, 15784 Athens, Greece
e-mail: dpanagop@biophysics.gr; dpanagop@biol.uoa.gr

D. J. Panagopoulos
Radiation and Environmental Biophysics Research Centre,
79 Ch. Trikoupi str., 10681 Athens, Greece

A. Karabarbounis
Department of Physics, Section of Nuclear and Particle Physics (A.K.), University of Athens, Athens, Greece

C. Liolioussis
Department of Physics, Section of Applied Physics, Electronics Laboratory (C.L.), University of Athens, Athens, Greece

Introduction

Modern man is constantly exposed to extremely low frequency (ELF) of 50–60-Hz electric and magnetic fields in residential environment [ELF is the region of electromagnetic spectrum with frequencies 0–300 Hz]. Electric energy is produced in the form of 50–60-Hz alternating

three-phase electric current and transported to residential areas by high-voltage power lines of usually hundreds of kV in order to minimize thermal loses. Within residential areas, the high voltage is transformed to 220–230 V prior to distribution for residential usage. Close to transformer substations or under power lines, the magnetic field intensity may reach values up to 0.2 G, while the electric field intensity may reach values exceeding 10 kV/m. Moreover, close to certain industrial or medical applications, people may be exposed to magnetic fields significantly stronger than 1G [1–3].

Several epidemiological studies during the last 30 years have shown a connection between exposure to power line or transformer fields and cancer [4–13]. This connection has been shown for magnetic field intensities down to 2 mG [7, 13] or distances from power lines up to 600 m [10]. Another epidemiological study points to the electric and not the magnetic component of the power line fields to have a connection with childhood leukaemia for intensities down to 10 V/m [14].

The current exposure limits for 50-Hz magnetic fields—for root-mean-square (*rms*) magnetic field intensities—are 1G (24-h exposure) for the general population and 10 G (few hours of exposure during the working day) for occupational exposure. The corresponding 50-Hz electric field exposure limits are 5 and 10 kV/m [15, 16]. These values are even higher than those usually accounted under power lines or close to transformers.

At the same time, all living organisms in the terrestrial environment are constantly exposed since the beginning of their existence to terrestrial static electric and magnetic fields of average intensities of $\sim$130 V/m and $\sim$0.5 G, respectively. Variations in the intensity of the terrestrial magnetic field on the order of $\pm$0.1 G during "magnetic storms" or "geomagnetic pulsations" mainly due to changes in solar activity are connected with increased rates of animal (and human) health incidents, including nervous and psychic diseases, hypertensive crises, heart attacks, cerebral accidents, and (consequently) mortality [17, 18]. Increase in solar activity leads to corresponding increases in intensity of visible, ultraviolet, gamma, and meson solar radiation; increase in ionization of the earth's atmosphere; increase in intensity of atmospheric discharges; and increases in the earth's magnetic and electric fields [18]. It is interesting to note that even human female fertility periodic variations seem to follow variations in the earth's magnetic and gravitational fields due to lunar periodic motion determined by the lunar month (28 days) [19].

Therefore, it seems that while living organisms adapt to constant values of natural static electric and magnetic fields, variations in these fields are responsible for increased biological and consequent health effects on living organisms. Since living organisms seem to perceive electromagnetic fields (EMF) as environmental stress factors [20], they can adapt more easily to them when their parameters are kept constant or vary slightly in time. In addition, living organisms do not seem to have defence mechanisms against large variations in natural EMFs, and thus it seems reasonable that even more they do not have defence mechanisms against unnatural (man-made) EMFs, which are mostly not static but varying in time (alternating and pulsed fields, modulated fields including simultaneously many different frequencies, etc.).

In the present study, we investigate the effect of ELF alternating magnetic field on a well-known biological model, the reproductive capacity of *Drosophila melanogaster*. Previous experiments with alternating or pulsed ELF magnetic field exposure of *Drosophila* flies have reported increased embryonic mortality and decreased oviposition with 17.6 and 10 G [21], induction of lethal mutations with 35 G [22], a slight but statistically significant increase in fertilized egg mortality with 50–400 mG [23], enhanced transcription with 3.8–35 G [24], or 6.16 % average decrease in reproductive capacity with 70 G [25]. In a more recent study, exposure of maternal and first filial generation (F_1) females to 50-Hz magnetic field reduced the oviposition of these insects in their subsequent generations [26]. Continuous exposure to 50-Hz, 0.5- or 5-mT (5 or 50 G) alternating magnetic field during 40 generations revealed a mutagenic action of this field [27]. Exposure of 3rd instar larvae to a more intense 50-Hz, 20-mT (200 G) alternating magnetic field for 24 h resulted in the eclosion of mutant flies due to enhancement of chromosomal recombination and non-disjunction. The authors suggested that the effects are not due to the magnetic field itself but actually due to the magnetically induced electric field/"eddy currents" [28]. In another study, exposure of 1st, 2nd, and 3rd instar larvae to a 50-Hz, 11 mT (110 G) alternating magnetic field for 2–8 h resulted in significant increase in morphological abnormalities in the developed adult flies, and the effect was positively correlated with the exposure duration [29]. Moreover, combination of 50-Hz sinusoidal 1G magnetic field and elevated temperature of 34–37 °C was found to affect strongly and adversely embryogenesis by producing morphological malformations on the embryos and delaying their development, at a considerably higher degree than with elevated temperature alone [30]. In addition to these results, several studies with negative results are also published: A non-significant statistically slight increase in recessive lethal mutation rate was reported after 13–14 days of exposure of adult flies to 500 μT (5 G) and 5 mT (50 G), 50-Hz magnetic field [31], and in an older study, no effect in chromosomal aberrations with 1 G was reported [32]. In a replication study of Ramirez et al.'s study [21], the authors reported no effect on oviposition nor on egg mortality [33]. No increase in developmental abnormalities and no

teratogenic action were found after exposure to 60-Hz, 1-G magnetic field [34].

In some of the above experiments aimed to study the effects of ELF magnetic fields on *Drosophila* reproduction [21, 23, 26, 33, 34], the procedures included counting of laid eggs under a stereo-microscope, an operation that encounters significant error. In addition, in those works they did not use newly emerged maternal–paternal flies, but instead flies that were collected from the general stock population [23, 26, 34] or flies 4–5 days old [21, 33]. Eggs from older flies have a considerable percentage of mortality. In those studies, they investigated effects on embryogenesis (egg mortality or teratogenesis). Embryogenesis seems to be a more resistant developmental stage than gametogenesis [25, 35] and therefore, it is better to study effects during gametogenesis rather than studying the same effects during embryogenesis (e.g. eggs die much more easily during oogenesis than during embryogenesis). In one study that reported no findings [34], there was heavy treatment of the embryos (with chemicals, dechorionation, homogenization, centrifugation, etc.), which can damage cells regardless of EMF exposure. Moreover, in their experiments with untreated embryos, in three out of four exposed groups there was a decrease in hatching compared with control group, which was not reported in the results. In contrast, they searched for morphological abnormalities in the hatched flies, which are unlikely to occur by magnetic field exposure alone at intensities up to 1 G. In two other studies [21, 33], only the female flies were exposed which were 4 and 5 days old, respectively, and already mated. Therefore, any effect on spermatogenesis or mating was excluded, and any effect on oogenesis was diminished. In both studies, there was no light periodicity but complete constant darkness which is unnatural for the flies. In addition, while the second study [33] aimed to replicate the first, there were different exposure temperatures (21 °C in [21], and 23 °C in [33]), and moreover, in the second study [33], humidity and food synthesis were not reported, and magnetic field intensity calculation was based on erroneous formula ($B = \mu_0 NI/R$ instead of $\mu_0 NI\sqrt{4\alpha^2 + R^2}$ as shown in 2.2).

Another weak point in the majority of previous experiments with magnetic field exposure [23, 24, 26–32, 34] was that they used Helmholtz coils. This type of coils is readily found in physics laboratories used to study uniform magnetic fields. Experiments with Helmholtz coils lack sham exposure in identical conditions other than magnetic field. Moreover, they are most usually available to create a horizontal magnetic field. Horizontally directed magnetic fields can be strongly affected by the terrestrial field which is quiet intense ($\sim$0.5 G or $\sim$50 μT). A small change in the direction/position of a horizontally oriented magnetic field may result in a large alteration in its magnitude due to interference with the terrestrial field which is of constant direction in a certain place. This goes also for studies that used cylindrical coils but horizontally oriented during the exposures [21]. In contrast, vertically oriented magnetic fields such as those created by cylindrical vertical coils are equally affected by the terrestrial field regardless of their orientation. Large, cylindrical coils are not readily found and need to be constructed. In our experiments with magnetic field exposures, we have addressed these points as described below (Sect. 2.2).

Finally, another limitation in the majority of the previous studies [21–24, 26, 27, 29–34] is that there was no estimation or measurement of the magnetically induced electric field, which always coexists with any time-varying magnetic field, and it is an important parameter of the resultant EMF.

In view of the above conflicting results and in an attempt to minimize errors, we used vertical cylindrical coils with well-defined exposure parameters. Moreover, we exposed newly eclosed maternal and paternal flies to study the effects during gametogenesis, and assessed reproductive capacity by the number of F_1 pupae instead of the number of laid eggs. The aim of the present study was to investigate whether ELF magnetic fields at environmental intensities or a little higher have adverse biological action. We investigated the biological action of the ELF fields on the reproductive capacity of *Drosophila melanogaster* which is a sensitive biological index, by use of a well-tested experimental protocol of ours used previously for different types of EMFs [25, 36–38]. Moreover, we investigated possible DNA damage on the reproductive cells during oogenesis by the use of the TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay as in previous experiments of ours with other types of EMFs [39–41].

Materials and Methods

Experimental Animals

Our experimental animals were *Drosophila melanogaster* flies, 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 % of relative humidity. The collected newly emerged adult flies for each experiment were separated into different groups within identical 50-ml cylindrical glass vials with 2.5 cm diameter and 10 cm height with equal amounts of food, as described extensively in previous reports of ours [25, 36–41].

The reproductive capacity of this insect—especially gametogenesis (oogenesis and spermatogenesis)—is a model biological system that is very well studied, with a very good timing of its developmental processes under certain laboratory conditions [35].

Following a well-tested protocol of ours, the reproductive capacity was defined by the number of F_1 pupae, which corresponds under the conditions of our experiments to the number of laid eggs (oviposition), 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 [35–41].

Exposure System

We designed and constructed two identical cylindrical coils for exposure (named L_1) and sham exposure (named L_2) of biological samples, respectively. The only difference between the two coils is that while the turns of L_1 are all parallel and in the same direction between them, in L_2 half of the turns are antiparallel (parallel but in opposite direction), so that the magnetic (and the magnetically induced electric) field over a region of about 12 cm width around the centre of the coil L_2 is zero. Since the two coils have identical geometrical characteristics (length, diameter, etc.) and equal number of turns of the same insulated wire, and given that the same current passes through the two coils, all other conditions except EMF (such as temperature, illumination, air flow, humidity, etc.) are identical within the two coils during the exposures. The only difference is that within L_1 there are certain values of magnetic and induced electric fields (as defined by the coil characteristics and the electric current intensity) while within L_2—and more specifically over the $\sim$12-cm region around the centre of L_2—both the magnetic and the induced electric fields are zero. Then, L_1 is used for magnetic field exposure, while L_2 is the corresponding control (used for sham exposure). The two coils were connected in series between them as well as to an adjustable alternating 50-Hz voltage supplier (S), a variable resistor, and an amperemeter, so that the same current—measured each moment by the amperemeter—flows through both of them. Specific characteristics of the coils are as follows: length $R = 0.25$ m, radius $\alpha = 7.5 \times 10^{-2}$ m, diameter of insulated wire $d = 2 \times 10^{-3}$ m, number of turns in each coil $N = 330$.

During the exposures/sham exposures, the glass tubes with the insects were suspended by nylon strings in the centre of the air cores of the coils L_1 and L_2. Photographs of the coils and the glass tubes within them can be found in [25]. After it was checked by preliminary experiments that the sequence (mutual position) of the two coils did not affect the outcome of the experiments, their positions were always the same during all experiments, at a certain place of the laboratory with the minimum stray 50-Hz fields and in at least 2 m distance between each other so that the fields of L_1 do not affect L_2.

According to the characteristics of the coils, theoretical calculation for the intensity of the magnetic field in the centre of the air core of L_1 gives:

$$B = \frac{\mu_0 IN}{\sqrt{4a^2 + R^2}} \tag{1}$$

where: $\mu_0 = 4\pi \cdot 10^{-7}$ V s/A m, the absolute magnetic permeability of vacuum or air, B (in T) the magnetic field intensity, I (in A) the electric current intensity flowing through the coil, N the number of turns, R the length of the coil, and a the radius of the turns (both in m).

By substituting the values of μ_o, N, R, a, in Eq. (1), we get:

$$\begin{aligned} &B = 14.22 \times 10^{-4} \times I, \quad (B \text{ in T},\ I \text{ in A}) \quad \text{or} \\ &B = 14.22 \times I \end{aligned} \tag{2}$$

(B in G, I in A). Equation (2) gives the magnetic field intensity in the centre of the air core of the coil L_1 in relation to the electric current intensity that flows through the turns of the coil.

Equation (2), which was theoretically deduced, was then verified experimentally by measuring the magnetic field intensity in the centre of L_1. Magnetic field intensity measurements within the coils were performed by use of a "Phywe Teslameter". The deviation between the theoretically calculated and the measured magnetic field intensity values was less than $\pm$ 0.025 G for all the field intensities used in the exposures.

By controlling the intensity of electric current through the variable resistor, we get the desirable values of magnetic field intensity in L_1. Thus, the exposure coil L_1 produces magnetic field with controllable parameters, while in the centre of the air core of the control coil L_2, the intensity of magnetic field (and of the magnetically induced electric field) is zero.

The magnetically induced electric field in L_1 along a circular path l of radius α, at the centre of L_1 vertical to the coil's axis, including a surface S is given by Maxwell's third equation:

$$\oint_l \vec{E}_{ind} \cdot \mathrm{d}\vec{l} = -\frac{d}{\mathrm{d}t} \int_S \vec{B} \cdot \vec{u}_N \mathrm{d}S \tag{3}$$

$\vec{B}, \vec{E}_{\mathrm{ind}}$, are the magnetic and the induced electric field intensities, respectively, $\mathrm{d}\vec{l}$ is an incremental length along the closed path l of induced electric field circulation, and $\vec{u}_N$ is the unit vector vertical to the surface S.

Assuming $\vec{E}_{\mathrm{ind}}$ is parallel to and independent of l, and $\vec{B}$ is vertical to and independent of S, Eq. (3) becomes: $E_{\mathrm{ind}} \oint_l \mathrm{d}l = -\frac{\mathrm{d}_B}{\mathrm{d}t} \int_S \mathrm{d}S$ which gives:

$$E_{\mathrm{ind(rms)}} = \pi v N B_{\mathrm{rms}} \alpha \quad (4)$$

($E_{\mathrm{ind(rms)}}$ is the root-mean-square value of the induced electric field in V/m, v in Hz, B_{rms} the root-mean-square value of the magnetic field in T, α in m).

It is important to state that the magnetically induced electric field is always naturally produced and coexisting with any time-varying magnetic field, and especially in the case of ELF fields, there is no way to totally eliminate it or insulate it by shielding. [Metal grids can reduce electric fields at a certain degree, but not totally eliminate them. For a significant decrease, a closed metal box is necessary.] All environmental alternating magnetic fields such as those originating from power lines, transformers, electric wiring, etc. include also an electric component. Performing experiments with a reduced/insulated electric component would not represent real conditions. The induced electric field has the same waveform (in our case sinusoidal-alternating) and the same frequency (in our case, 50 Hz) with the magnetic one that generates it as denoted by Eq. (4). The two fields have a phase difference of $\pi/2$ between them. Thus, in reality there is never any pure exposure to a time-varying magnetic field without a simultaneous exposure to a corresponding induced electric one.

The opposite does not occur: in the case of a time-varying electric field, the corresponding induced magnetic one is given by the second term of the second part of Maxwell's fourth equation:

$$\oint_l \vec{B} \cdot \mathrm{d}\vec{l} = \mu_0 I + \varepsilon_0 \mu_0 \frac{\mathrm{d}}{\mathrm{d}t} \int_S \vec{E} \cdot \vec{u}_N \mathrm{d}S \quad (5)$$

More specifically, the induced magnetic field due to the temporal variation in the corresponding electric one is:

$$\oint_l \vec{B}_{\mathrm{ind}} \cdot \mathrm{d}\vec{l} = \varepsilon_0 \mu_0 \frac{\mathrm{d}}{\mathrm{d}t} \int_S \vec{E} \cdot \vec{u}_N \mathrm{d}S \quad (6)$$

$\varepsilon_0 = 8.854 \cdot 10^{-12}$ C^2/N m^2, the absolute permittivity of vacuum.

As denoted by Eq. (6), the induced magnetic field is usually of negligible intensity due to the small values of the constants ε_0 and μ_0 that are included in this equation.

Any biological effect produced by a combination of the two coexisting fields is still unknown whether is due to the magnetic or to the corresponding induced electric field or due to the combination of both. Yet, the vast majority of the studies seem to ignore the induced electric field and concentrate only on the magnetic component.

In the present experiments, we exposed the insects to three different magnetic field intensities: 1 G (=0.1 mT), which is close to a maximum intensity that may be accounted below power lines and the limit for the general public exposure; 11 G (=1.1 mT), which is very close to the occupational exposure limit; and 21 G (=2.1 mT), to test the effect of a stronger field. For every exposed group at any of the three different intensities, there was a separate identical sham-exposed group.

The characteristics of both the magnetic and the magnetically induced electric fields within the exposure coil L_1 are listed in Table 1. The temperature in the air cores of both coils during the exposures was identical between them, but slightly different for the three different electric current (and EMF) intensities. It was determined by the electric current flowing through the coil turns and was controlled also by the air cooler of the room. The corresponding temperatures for the three different sets of EMF intensities are also given in Table 1.

Exposure Procedure

In each experiment, we collected newly eclosed adult flies from the stock, anesthetized them lightly with diethyl ether and separated them into two identical different groups—one exposed and one sham-exposed—following the same methodology as described previously [36]. Each one of the two groups in each experiment consisted of ten females and ten males, newly emerged flies, as in previous experiments [36–40].

We exposed the flies within the cylindrical glass vials with their food. The sham-exposed groups received identical treatment to the exposed ones, except that they were exposed to zero fields during the sham exposures within the L_2 coil, but in identical other conditions with the exposed ones, such as temperature, light, humidity, etc.

Table 1 EMF characteristics

Parameter	Magnetic field	Magnetically induced electric field	Electric current in coils	Temperature within coils
Intensity (rms)	$B_1 = 1$ G (0.1 mT)	$E_1 = 0.13$ V/m	0.07 A	24.8 ± 0.5 °C
	$B_2 = 11$ G (1.1 mT)	$E_2 = 1.43$ V/m	0.77 A	25.3 ± 0.5 °C
	$B_3 = 21$ G (2.1 mT)	$E_3 = 2.72$ V/m	1.48 A	25.5 ± 0.5 °C
Frequency	50 Hz	50 Hz	50 Hz	–
Direction	Vertical	Horizontal	Helical	–
Shape	Sinusoidal-alternating	Sinusoidal-alternating	Sinusoidal-alternating	–

We performed twelve experiments for each different magnetic (and corresponding induced electric) field intensity to investigate the effect of the ELF field on reproductive capacity. In five of them, for each set of field intensities, we performed the TUNEL assay as well. Then, we had three series of experiments (one for each of the three different sets of intensities) with twelve experiments each. The exposures took place for a total of 5 days in each experiment starting on the day of eclosion, 1 h after the insects were awaken from the first anaesthesia [36].

In the first series of experiments (1.1–1.12), the insects were exposed to 1-G magnetic field intensity. In the second series of experiments (2.1–2.12), they were exposed to 11-G magnetic field intensity, and in the third series (3.1–3.12), they were exposed to 21 G. At the same time, the samples were automatically and simultaneously exposed to the corresponding intensities of the magnetically induced electric field 0.13, 1.43 and 2.72 V/m (Table 1).

During the first 48 h of each experiment, the males and females of each group were kept and exposed/sham exposed into separate glass vials (two separate vials for each exposed/sham-exposed group, four vials in each experiment). 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 [35, 42]. Keeping males separately from females for the first 48 h of each experiment ensures that the flies are in complete sexual maturity and ready for immediate mating and laying of fertilized eggs [36]. This ensures that all laid eggs during the next 72 h will be fertilized and helps to decrease variability in oviposition counts.

After the first 48 h, the males and females of each group (twenty flies) were lightly anesthetized again and put together (ten pairs) within the same vial (one vial for each exposed/sham-exposed group in each experiment) to be 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.

Reproductive Capacity Assessment

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

The female maternal flies that were removed from the glass vials were immediately anesthetized again, and their ovaries were dissected and fixed for the TUNEL assay as described below (2.5).

After the six additional days that the vials without the maternal–paternal flies were kept in the culture room without exposure, most F_1 embryos (deriving from the fertilized laid eggs) were in the stage of pupation, where they clearly could be seen macroscopically and easily counted without error on the walls of the glass tubes. Nevertheless, the counting was blinded (the person who counted did not know the origin of the sample). As we have explained already, under these conditions the number of F_1 pupae—coming from newly emerged flies within the first 3 days of maximum oviposition—coincides with the number of laid eggs [36]. Hence, by counting the F_1 pupae 11–12 days after the beginning of each experiment, we get a representative estimate of each group's reproductive capacity. In this way, instead of counting eggs on the surface and within the food, which encounters considerable error, we count pupae on the walls of the glass tubes with no error at all. That was a simple but important innovation of ours [36].

TUNEL Assay

In order to investigate the ability of the ELF magnetic field to damage DNA during early and mid-oogenesis, we used the TUNEL assay. This assay is a marker for DNA fragmentation (severe DNA damage including both single- and double-strand DNA breaks). Ovaries were dissected in Ringer's solution and separated into individual ovarioles from which the egg chambers of stages 11–14 were taken away as previously. In egg chambers of stages 11–14, programmed cell death takes place normally in the nurse and follicle cells [43–45]. Therefore, we kept and treated ovarioles and individual egg chambers from germarium up to stage 10. As described in [44], samples were fixed in phosphate-buffered saline (PBS) solution containing 4 % formaldehyde plus 0.1 % Triton X-100 (Sigma Chemical Co., Munich, Germany) for 30 min and then rinsed three times and washed twice in PBS for 5 min each. Then, samples were incubated with PBS containing 20 μg/ml proteinase K for 10 min and washed three times in PBS for 5 min each. For in situ detection of fragmented genomic DNA, samples were incubated in TUNEL-mix (Boehringer Mannheim kit, Boehringer Mannheim Corp., Indianapolis, IN, USA), containing fluorescein dUTP and transferase (50 μl TUNEL-mix contained 45 μl TUNEL-label (fluorescein dUTP) and 5 μl transferase). Samples were incubated for 3 h at 37 °C in the dark and then washed six times in PBS for 1 h and 30 min in the dark (two times for 10 min each, two times for 15 min each, and two times for 20 min each) and finally mounted in antifading mounting medium (90 % glycerol containing 1.4-diazabicyclo (2.2.2) octane (Sigma Chemical Co.)) to prevent from fading and 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.

Statistical Analysis

The results on reproductive capacity and cell death (DNA fragmentation) induction were analysed statistically by single factor analysis of variance test (Microsoft Excel statistical program), which calculates the probability (P) that differences between groups are due to random variations. The smaller this probability is, the more significantly the groups differ between them (in their reproductive capacity or in the percentages of TUNEL-positive egg chambers). A probability level of $P < 0.05$ was accepted as statistically significant. In addition, (Pearson's) correlation analysis was performed between reproductive capacity and magnetic or induced electric field intensity, as well as between ovarian DNA fragmentation induction and magnetic or induced electric field intensity, in order to get an estimation of which component (the magnetic field, or the magnetically induced electric field) might be more responsible for the effects (IBM SPSS statistical program) [46, 47].

Results

Mean numbers of F_1 pupae from the exposed and sham-exposed groups in 12 replicate experiments with each different set of EMF intensities, average means with standard deviation (SD), and P values between corresponding exposed and sham-exposed groups are listed in Table 2.

The average mean number of F_1 pupae $\pm$ SD (representing reproductive capacity) of the exposed and sham-exposed groups for the three sets of experiments corresponding to the three different field intensities is represented graphically in Fig. 1.

The results on Table 2 and Fig. 1 show that the number of F_1 pupae—a representative index of the insect's reproductive capacity—is decreased due to the ELF EMF exposure and the effect increases with increasing field intensity. For $B_1 = 1$ G, $E_1 = 0.13$ V/m, the average decrease in 12 experiments was 2.94 %. This decrease is not statistically significant ($P = 0.099$). The decrease in reproductive capacity becomes larger (3.73 %) and statistically significant ($P < 0.05$) after exposure to a more intense magnetic (and magnetically induced electric) field, $B_2 = 11$ G, $E_2 = 1.43$ V/m. Finally with an even more intense magnetic (and induced electric) field $B_3 = 21$ G, $E_3 = 2.72$ V/m, the decrease becomes even larger (4.3 %) and even more statistically significant ($P < 0.03$) (Table 2).

The application of the TUNEL assay, in five replicate experiments with each set of EMF intensities, revealed that the decrease in reproductive capacity is due to severe DNA damage and consequent cell death in the insect's reproductive cells, induced by the ELF field. The differences in percentages of egg chambers with a TUNEL-positive signal (denoting DNA fragmentation) between exposed and corresponding sham-exposed groups (listed in Table 3) were even higher and even more statistically significant than—but in close correlation with—the corresponding percentages of reproductive decrease. In the five replicate experiments with each set of EMF intensities, the average increase in the percentage of egg chambers with DNA fragmentation was for (B_1, E_1): 5.72 % ($P < 0.0007$), for (B_2, E_2): 6.71 % ($P < 0.0006$), and for (B_3, E_3): 7.52 % ($P < 0.0004$) (Table 3). The results of the application of the TUNEL assay listed in Table 3 regarding induction of DNA fragmentation and consequent cell death/egg chamber elimination by the ELF EMF are graphically represented in Fig. 2.

Figure 3 shows an ovariole of a sham-exposed insect containing egg chambers from germarium to stage 9, all TUNEL negative. This was a representative picture of ovarioles and single egg chambers from sham-exposed insects. The number of egg chambers with a TUNEL-positive signal was less than 7 % in the sham-exposed female insects, and in all these cases, the DNA fragmentation was observed in one of the two checkpoints, that is, the germarium or the stages 7–8, in absolute agreement with previous studies [39–41].

Figures 4, 5, and 6 show ovarioles from exposed female insects with TUNEL-positive signal in one of the two or in both checkpoints. Figure 7 shows a stage 8 egg chamber from an exposed female with a TUNEL-positive signal in all three kinds of egg chamber cells. In all samples of ovarioles and individual egg chambers of insects exposed to the ELF field and from all three sets of experiments, the DNA fragmentation was observed exclusively at one of the two or in both checkpoints, and there was no TUNEL-positive signal (DNA fragmentation) observed at any other developmental stages of early and mid-oogenesis than germarium and stages 7–8 (exceptions were less than 1 %).

In contrast to our previous studies with microwave radiation [39–41] in which the germarium checkpoint was found to be more sensitive than the stage 7–8, in the present study the mid-oogenesis checkpoint (stage 7–8) was found to be more sensitive to the ELF magnetic field exposure than the germarium for the three different intensity values tested. For insects exposed to B_1, E_1, the ratio of TUNEL-positive to total number of stage 7–8 egg chambers was 0.458 while the ratio of TUNEL-positive to total number of germaria was 0.317. The corresponding ratios for insects exposed to B_2, E_2 were 0.512 and 0.286, and for insects exposed to B_3, E_3, they were 0.515 and 0.306 (Table 3, column 3).

Table 2 Effect of different ELF magnetic and magnetically induced electric field intensities on reproduction

EMF	Experiment no	Mean number of F_1 pupae per maternal fly (exposed)	Mean number of F_1 pupae per maternal fly (sham exposed)	Deviation from sham-exposed groups	*P* value, between exposed and sham-exposed groups
	1.1	13.9	14.3		
	1.2	12.2	12.6		
	1.3	14	14.7		
	1.4	12.9	13		
	1.5	12.6	12.9		
	1.6	13.3	13.7		
	1.7	13.6	14		
	1.8	13	13.2		
	1.9	12.9	13.5		
	1.10	13.3	14.1		
	1.11	13.4	13.7		
	1.12	13.2	13.4		
B_1, E_1	Average ± SD	13.19 ± 0.52	13.59 ± 0.62	−2.94 %	0.099
	2.1	12.8	13.6		
	2.2	13.4	13.8		
	2.3	13.4	14.1		
	2.4	14.2	14.6		
	2.5	13	13.5		
	2.6	12.9	13.7		
	2.7	13.7	14.4		
	2.8	12.7	13.3		
	2.9	12.5	12.9		
	2.10	13.1	13.2		
	2.11	14	14.2		
	2.12	12.2	12.7		
B_2, E_2	Average ± SD	13.16 ± 0.60	13.67 ± 0.59	−3.73 %	<0.05
	3.1	12.5	13.3		
	3.2	13.1	13.2		
	3.3	12.9	13.8		
	3.4	13.4	13.9		
	3.5	12.5	13		
	3.6	14.2	14.6		
	3.7	12.7	13.5		
	3.8	12.8	13.6		
	3.9	13.9	14.2		
	3.10	13.5	14.4		
	3.11	12.2	12.7		
	3.12	13.7	14.3		
B_3, E_3	Average ± SD	13.12 ± 0.60	13.71 ± 0.62	−4.3 %	<0.03

Although in the most egg chambers with DNA fragmentation the TUNEL-positive signal was observed mainly in the nurse cells (NC) and in less cases also in the follicle cells (FC), there were a few occasions (about 8 % of the total number of stage 7–8 egg chambers in all three sets of experiments with a TUNEL-positive signal in any type of egg chamber cells) that a TUNEL-positive signal was observed also in the oocyte (OC) of the stage 7–8 egg chambers (Fig. 7). In all these occasions, there was a TUNEL-positive signal also in the nurse and follicle cells. The number of these occasions increased with increasing field intensities (Table 3, column 7).

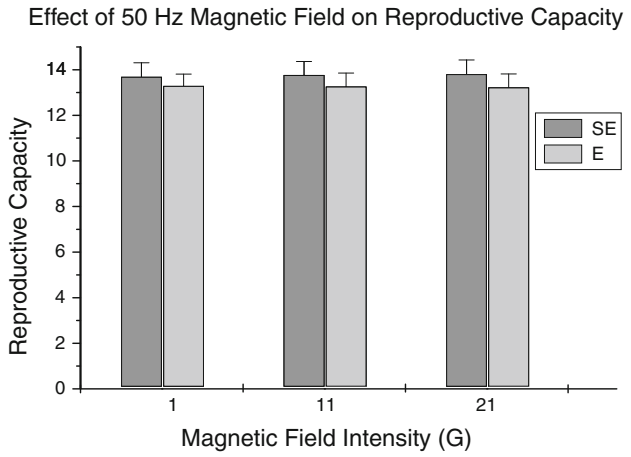

Fig. 1 Average reproductive capacity (average mean number of F_1 pupae per maternal insect) ± SD of sham-exposed (SE) and exposed (E) groups in twelve replicate experiments for three different magnetic field intensities: 1, 11, and 21 G

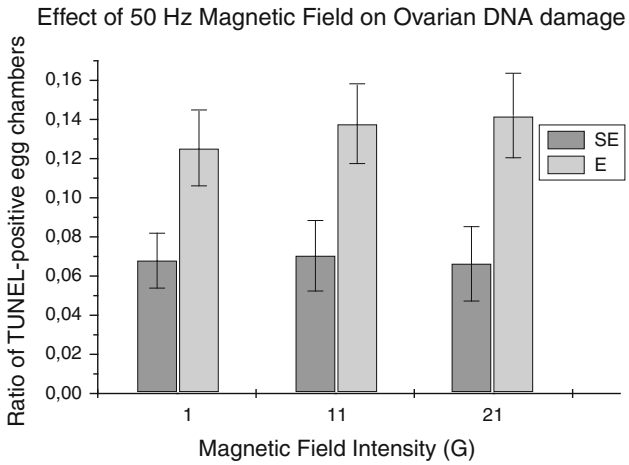

Fig. 2 Sum ratio of ovarian DNA fragmentation (number of TUNEL-positive to total number of egg chambers) for sham-exposed (SE) and exposed (E) groups, averaged over five replicate experiments ± SD, for the three different magnetic field intensities: 1, 11, and 21 G

Table 3 Effect of different ELF magnetic and magnetically induced electric fields intensities on ovarian DNA fragmentation

EMF	Developmental stages	Sum ratio of TUNEL-positive to total number of egg chambers (exposed) in five replicate experiments	Sum ratio of TUNEL-positive to total number of egg chambers (sham-exposed) in five replicate experiments	Difference from sham-exposed groups	*P*-value, between exposed and sham-exposed groups	Ratio of TUNEL-positive oocytes to total number of TUNEL-positive egg chambers at stage 7–8 (exposed)
	Germarium	20/63 = 0.317	14/57 = 0.246			
	7–8	81/177 = 0.458	39/164 = 0.238			6/81 = 0.074
	1–6, 9–10	7/629	2/599			
B_1, E_1	Sum ratio in all stages ± SD	108/869	55/820	+5.72 %	<0.0007	
		=0.1243 ± 0.019	=0.0671 ± 0.014			
	Germarium	14/49 = 0.286	10/52 = 0.1923			
	7–8	82/160 = 0.512	37/153 = 0.2418			6/79 = 0.076
	1–6, 9–10	5/530	1/485			
B_2, E_2	Sum ratio in all stages ± SD	101/739	48/690	+6.71 %	<0.0006	
		=0.1367 ± 0.02	=0.0696 ± 0.018			
	Germarium	22/72 = 0.306	13/68 = 0.191			
	7–8	86/167 = 0.515	36/171 = 0.211			7/86 = 0.081
	1–6, 9–10	6/571	4/570			
B_3, E_3	Sum ratio in all stages ± SD	114/810	53/809	+7.52 %	<0.0004	
		=0.1407 ± 0.021	=0.0655 ± 0.019			

Correlation analysis gave a slightly higher (although not significant statistically) correlation between the induced electric field intensity and the reproductive decrease (linear correlation coefficient $r = 0.9961$, $P = 0.056$) than between magnetic field intensity and reproductive decrease ($r = 0.9959$, $P = 0.058$). Similarly, ovarian DNA fragmentation was slightly better, but moreover significantly correlated with the induced electric field ($r = 0.9985$, $P = 0.035$) than with the magnetic ($r = 0.9983$, $P = 0.037$). Thus, while the slightly higher correlation with the electric field is not statistically significant at the 0.05 level with regard to the reproductive decrease, it is significant with regard to the ovarian DNA damage ($P < 0.04$).

We note that the reproductive capacity of the sham-exposed groups increased slightly with increasing field intensities (and with increasing temperature) (Tables 1, 2). Nevertheless, both the exposed and the sham-exposed groups in each experiment were under identical temperature and room conditions.

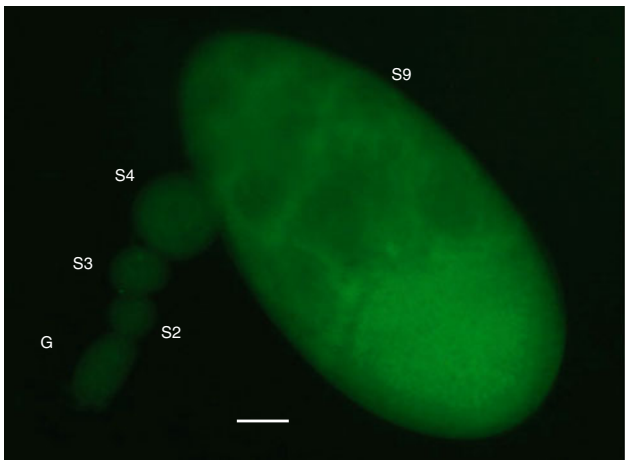

Fig. 3 Normally developed ovariole of a sham-exposed female Drosophila insect, containing egg chambers from germarium up to stage 9, all TUNEL-negative. *Bar* 10 μm

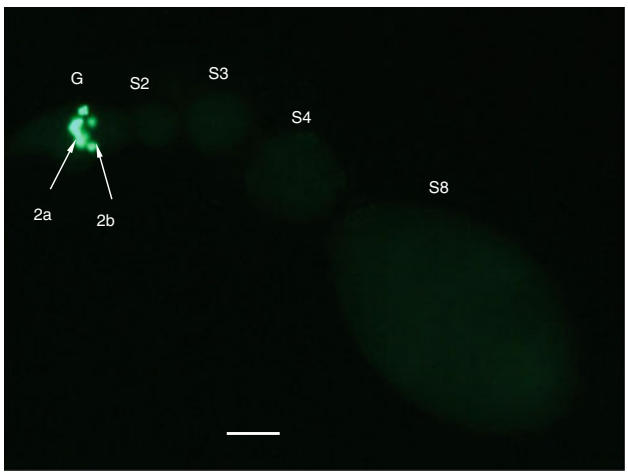

Fig. 4 Ovariole of an exposed female Drosophila insect, containing egg chambers from germarium up to stage 8, with DNA fragmentation only at the germarium (region 2a/2b) and TUNEL-negative at all other developmental stages (S1–S8). *Bar* 10 μm

Discussion and Conclusions

The results of the present study show that residential 50–60-Hz magnetic fields decrease insect reproduction by severe DNA damage (DNA fragmentation) induction in the reproductive cells. The effect on reproductive capacity is small (up to 3 % decrease) at environmentally accounted intensities (up to 1 G, 0.13 V/m). Although this percentage is not statistically significant ($P = 0.099$) with 12 replicate experiments, it becomes significant if we repeat the experiment more times (e.g. with 12 more experiments of the same outcomes, it becomes $P < 0.02$). Thus, the effect, although small, is replicable and actually statistically significant. The effect on reproductive capacity becomes

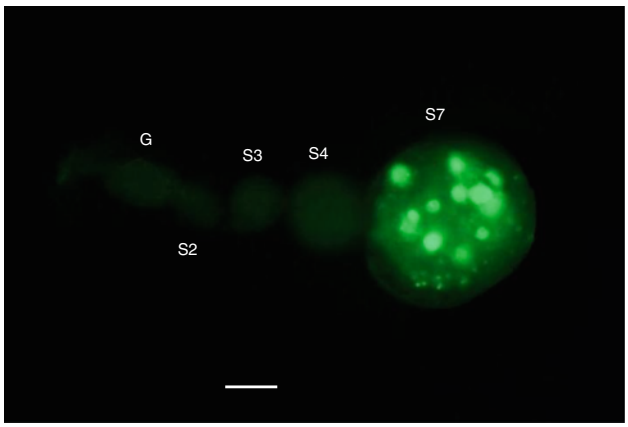

Fig. 5 Ovariole of an exposed female Drosophila insect, containing egg chambers from germarium up to stage 7, with DNA fragmentation only at the mid-oogenesis checkpoint (stage 7) and TUNEL-negative at all other developmental stages. *Bar* 10 μm

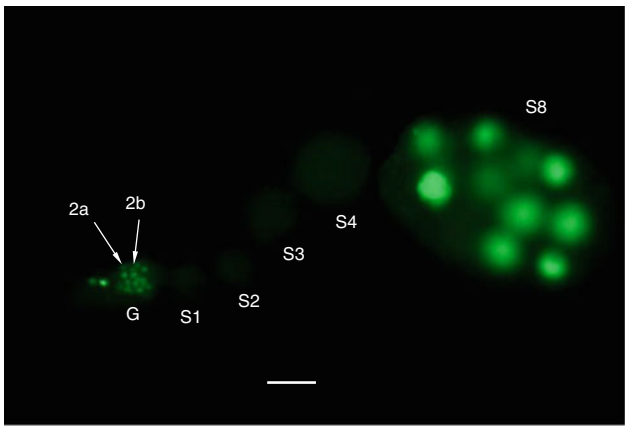

Fig. 6 Ovariole of an exposed female Drosophila insect, containing egg chambers from germarium up to stage 8, with DNA fragmentation at the two checkpoints, germarium (region 2a/2b) and stage 8, and TUNEL-negative at all intermediate stages. *Bar* 10 μm

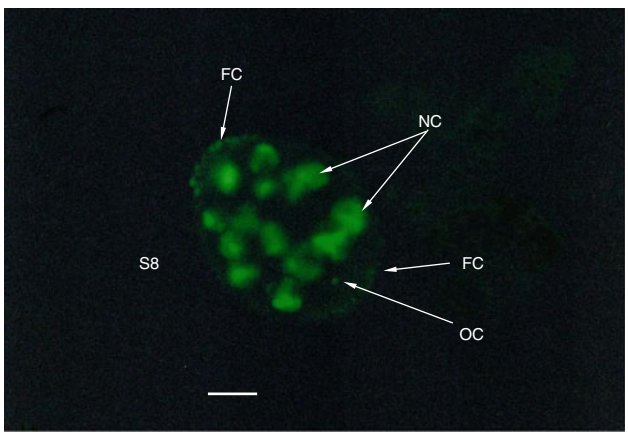

Fig. 7 A stage 8 egg chamber of an exposed insect with DNA fragmentation (severe DNA damage) at all three types of egg chamber cells, nurse cells (NC), follicle cells (FC), and the oocyte (OC). *Bar* 10 μm

larger and more significant statistically with increasing field intensities. The effect on DNA fragmentation induction (as revealed by the application of the TUNEL assay) is even larger, and for this reason, it is statistically significant with all field intensities tested. In any case, the effect of DNA fragmentation induction is in close correspondence with the ultimate effect on reproductive capacity. The fact that the effect on DNA fragmentation is more significant than the ultimate effect on oviposition is in agreement with our previous studies regarding microwave exposure of the same biological system, in which the effect on egg chamber elimination was always larger than the ultimate effect on oviposition (reproductive capacity). This might be due to some unknown mechanism by which the reproductive system of *Drosophila* compensates the loss of damaged egg chambers by producing new ones, possibly at a higher rate during an insult's presence.

The fact that DNA fragmentation was observed exclusively at the two checkpoints and not at all the developmental stages of early and mid-oogenesis as with microwave exposure shows that ELF magnetic field is probably a milder environmental stress factor than microwave radiation.

The induced DNA fragmentation in the reproductive cells was observed in all three kinds of egg chamber cells, that is, in the nurse cells, in the follicle cells, and in the oocyte. Induced DNA fragmentation in the oocyte was previously observed only after microwave exposure [39–41] and not after exposure to other insults previously tested such as starvation, or cytotoxic chemicals [44, 48]. Therefore, it is an important novel finding that residential ELF EMFs can induce DNA fragmentation in the oocyte. While damage in the nurse and follicle cells during early or mid-oogenesis will result in the degeneration/elimination of the egg chamber and consequently in a reproductive decrease, a damage in the oocyte's genome may result—if not to cell death/egg chamber elimination—in inheritable mutations transferred to the next generations. Certainly, in all cases where a TUNEL-positive signal was observed in the oocyte, there was a corresponding TUNEL-positive signal in the other cell types as well, while the opposite did not occur. The oocyte is indeed more protected (as it has smaller nucleus than the nurse cells, and additionally, it is surrounded/protected by the follicle cells, plus by large amounts of yolk proteins during mid- and late oogenesis) and probably more resistant to radiation as well. Thus, in all cases during early and mid-oogenesis that a DNA damage in the oocyte occurs, it is likely that the whole egg chamber will be eliminated by induced cell death after DNA damage in the other cell types. Nevertheless, in cases that the egg chamber is not eliminated and proceeds to late oogenesis where the nurse and follicle cells undergo programmed cell death anyway, a damage in the oocyte's genome may result in inheritable mutations transferred to the next generations. Such a possibility cannot be overlooked. This is perhaps an important issue for future experiments.

While after exposure to microwave radiation in our previous studies [39–41] the early oogenesis checkpoint (germarium) was found to be more sensitive than the stage 7–8 (mid-oogenesis checkpoint), in the magnetic field exposure of the present study the mid-oogenesis checkpoint was observed to be more sensitive. This is another novel finding of the present study that may be due to the probable fact that the ELF magnetic field represents a milder stress factor than microwave radiation. A possible explanation may be that at the beginning of the egg chamber development (germarium) the organism cotes the mild external insult in view of a possibility that physiological development may occur in spite of the insult's presence. When the stress persists and the egg chamber cannot develop normally, the organism itself eliminates it at the next checkpoint.

The consistent effect at both checkpoints (different stages of oogenesis) and in all three kinds of egg chamber cells probably shows that the underlying biophysical/biochemical mechanism for DNA damage after exposure to ELF fields is the same for different cell types (i.e. NC and OC which are germ cells and FC which are somatic cells) and at different stages of cell development and differentiation. This may be important in terms of extrapolating the present results to other organisms as well. Probably the effect of the EMF is a direct effect at tissue level (on the developing egg chambers), but indirect at cellular level (i.e. not directly on DNA but through the disruption of the cell's electrochemical balance) as discussed below.

The slight increase in reproductive capacity of the SE groups with increasing field intensities can be attributed to the slight corresponding increase in temperature due to the higher current intensity. This is in agreement with previous studies showing that oviposition increases with corresponding temperature increase within normal values [49]. Nevertheless, since both the exposed and sham-exposed groups in each experiment were under identical temperature between them, the above-described effect of DNA damage in the ovarian cells was induced non-thermally.

The present study showed that 50-Hz magnetic field induces severe DNA damage for intensities at least down to 1 G (= 0.1 mT) (which is the current ICNIRP limit for the general population exposure) and for induced electric field intensities at least down to 0.13 V/m, which is almost forty thousand times lower than the corresponding 50-Hz electric field exposure limit (5 kV/m). Our results are in agreement with the majority of the previous studies that investigated effects on *Drosophila* after exposure to ELF magnetic fields and found positive results [21–30]. Although the effects on insects might not be directly extrapolated to humans, we

consider that the results of the present study imply the need for reconsideration of the current ICNIRP exposure criteria as well as the need for minimizing human and animal exposure to these fields by keeping power lines and substations at safe distances from houses and work places. Although magnetic field intensities in living environments are usually much lower than 1 G, under high-voltage power lines, close to power substations, or close to certain industrial and medical applications, the fields may reach values close to or even significantly exceeding the current ICNIRP limits [1–3, 15, 16].

DNA damage induction is in every case an alarming finding for public health since—if not properly repaired by the organism's compensatory systems—it is the initiator of either cancer (in somatic cells other than neural), degenerative neural diseases such as Parkinson's or Alzheimer's in brain cells after corresponding cell death, or inheritable mutations (when occurs in the gametes and will not result in cell death/reproductive decrease). Severe DNA damage as is DNA fragmentation detected by the TUNEL assay is unlikely to be repaired.

The fact that the effect of DNA fragmentation (and the corresponding reproductive decrease) is slightly better correlated with the induced electric field intensity than with the magnetic field might indicate that the magnetically induced electric field may be more responsible than the magnetic itself for the recorded biological/health effects. This might also be an important finding in agreement with some earlier reports [14, 28, 50, 51] and also with the mechanism for the action of EMFs on cells that we have proposed [52, 53]. According to this theory, also called "Ion Forced-Vibration" mechanism, oscillating EMFs can alter cellular function by irregular gating of cation channels on the cell membranes. This theory shows that interaction between an external oscillating electric field and moving free ions within a membrane channel is stronger than interaction between external magnetic field of the same frequency and those ions, resulting in stronger biological activity of an electric field than of a corresponding magnetic one. Nevertheless, the two component fields may act independently of each other and the effects may be additive. Therefore, the present study in connection with the Ion Forced-Vibration theory and some of the previous experimental and epidemiological findings may constitute an indication that future biological studies should perhaps be oriented more on the effects of the ELF electric fields on biological systems than those of magnetic ones. In any case, we consider that future studies with ELF EMFs should focus not only on the magnetic but also on the electric component of the field.

Although an association between ELF EMFs and cancer has been consistently indicated by the majority of epidemiological studies during the last 30 years, one reason that this has not yet resulted in a reconsideration of the current exposure criteria is the alleged lack of a commonly accepted mechanism of action of such fields at cellular level [8] and the conflict among results of different biological studies [15]. Since the present study has shown that these fields induce severe DNA damage—which is the main initiator for cancer—for intensities at least down to 1-G magnetic fields or at least down to 0.1 V/m electric fields, we believe it is an important contribution to the literature concerning this matter. Moreover, the mechanism that we have previously proposed [52, 53] explains how ELF magnetic fields down to 1 G or electric ones down to 0.001 V/m can disrupt cellular function by altering intracellular concentrations of critical ions such as calcium, sodium, potassium, etc., which may in turn give a false signal for irregular release of free radicals or hydrolytic enzymes able to damage DNA. This is a plausible scenario for indirect action of external ELF fields on cells. According to the same mechanism, the effects increase with increasing field intensities, and this explains both the results of the present study and those of the epidemiological studies which indicate increased risk with increasing intensities/decreasing distances from, for example, power lines. Therefore, a lack of the explanation of epidemiological/biological findings does not any longer exist.

In conclusion, we believe that the present study, in agreement with the epidemiological studies and the majority of previous ELF/*Drosophila* biological studies, indicates a probable carcinogenic action of ELF EMFs and, therefore, a need for minimizing human/animal exposure to these fields by setting more stringent exposure criteria.

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