

Transgenic nematodes as biomonitors of microwave-induced stress

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Abstract

Transgenic nematodes (*Caenorhabditis elegans* strain PC72), carrying a stress-inducible reporter gene (*Escherichia coli* β -galactosidase) under the control of a *C. elegans hsp16* heat-shock promoter, have been used to monitor toxicant responses both in water and soil. Because these transgenic nematodes respond both to heat and toxic chemicals by synthesising an easily detectable reporter product, they afford a useful preliminary screen for stress responses (whether thermal or non-thermal) induced by microwave radiation or other electromagnetic fields. We have used a transverse electromagnetic (TEM) cell fed from one end by a source and terminated at the other end by a matched load. Most studies were conducted using a frequency of 750 MHz, at a nominal power setting of 27 dBm. The TEM cell was held in an incubator at 25°C inside a shielded room; corresponding controls were shielded and placed in the same 25°C incubator; additional baseline controls were held at 15°C (worm growth temperature). Stress responses were measured in terms of β -galactosidase (reporter) induction above control levels. The time-course of response to continuous microwave radiation showed significant differences from 25°C controls both at 2 and 16 h, but not at 4 or 8 h. Using a 5 × 5 multiwell plate array exposed for 2 h, the 25 microwaved samples showed highly significant responses compared with a similar control array. The wells most strongly affected were those in the rows closest to the source, whereas the most distant row did not rise above control levels, suggesting a shadow effect. These differential responses are difficult to reconcile with general heating effects, although localised power absorption affords a possible explanation. Experiments in which the frequency and/or power settings were varied suggested a greater response at 21 than at 27 dBm, both at 750 and 300 MHz, although extremely variable responses were observed at 24 dBm and 750 MHz. Thus, lower power levels tended, if anything, to induce larger responses (with the above-mentioned exception), which is opposite to the trend anticipated for any simple heating effect. These results are reproducible and data acquisition is both rapid and simple. The evidence accrued to date suggests that microwave radiation causes measurable stress to transgenic nematodes, presumably reflecting increased levels of protein damage within cells (the common signal thought to trigger *hsp* gene induction). The response levels observed are comparable to those observed with moderate concentrations (ppm) of metal ions such as Zn²⁺ and Cu²⁺. We conclude that this approach deserves further and

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more detailed investigation, but that it has already demonstrated clear biological effects of microwave radiation in terms of the activation of cellular stress responses (*hsp* gene induction). © 1998 Elsevier Science B.V.

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1. Introduction

There has been considerable media interest in the possible biological effects of continuous or pulsed exposure to microwave radiation, particularly at the frequencies used, e.g., for mobile phones. Unfortunately, the copious literature on microwave effects and those of lower-frequency electromagnetic fields (EMFs; reviewed in Ref. [1]) is confused by a wide range of very different experimental approaches (some of which are highly complex and difficult to reproduce in other laboratories; [1]), by methodological inconsistencies, and in some cases, by contradictory results (despite using similar conditions in the different studies). There are several possible sources of artefact in such studies, and there is often no clear molecular mechanism to explain the effects observed, which may occur only within narrow windows of frequency and/or power level and/or pulse length/interval and/or total exposure time [1].

Recent evidence has suggested that low-frequency EMFs may induce a classic stress response in cells [2], involving the induction of heat-shock (*hsp*) genes. These genes encode heat-shock proteins (HSPs) which function mainly as molecular chaperones in protein transport and in protein folding/unfolding/refolding within the cell. Stressors such as heat, heavy metals and oxidative stress all cause damage to cellular proteins, and induced HSPs counteract the accumulation of such damaged proteins by recycling those that can be rescued and removing those that are irreparably damaged for ubiquitination and proteolysis [3]. Overall, the heat-shock response represents a general mechanism for coping with increased protein damage while cells or organisms are under sublethal stress [4]. Protein damage appears to be the common signal that elicits the activation of most stress-inducible *hsp* genes, whereas other members of the *hsp* gene families are constitutively expressed in all cells and fulfil the routine requirements for protein folding and transport under normal (unstressed) conditions. The stress-inducible *hsp*

genes are activated by a common mechanism involving a heat-shock transcription factor (HSF) which interacts with multiple heat-shock elements (consensus NGAAN) in the promoters of these genes. In unstressed cells in higher organisms, HSF exists as a monomer which is not bound to the DNA; heat or other stresses promote trimerisation of the HSF with consequent binding to HSE arrays and transcriptional activation of the associated stress-inducible *hsp* genes [5,6].

Increased HSP production has been proposed as a useful biomarker of sublethal stress, since a large variety of stressors can act as inducers (reviewed in Refs. [4,7]). However, direct measurements of HSP proteins (e.g., using immunoblotting or metabolic labelling) are technically demanding, and background expression of constitutive *hsp*-related genes (also called heat-shock cognates) must be accounted for [8]. An alternative approach is to use transgenic test organisms carrying an *hsp*-gene promoter linked to a reporter gene whose product can be easily detected (such as β -galactosidase from the *Escherichia coli lacZ* gene). For this purpose, the free-living soil nematode *Caenorhabditis elegans* affords an ideal candidate, since it is small (ca. 1 mm adult size), genetically amenable to transgenesis [9], has a rapid life cycle (ca. 4 days at 20°C), and reproduces largely by hermaphrodite self-fertilization [10]. Two studies have shown that transgenic *C. elegans* strains, carrying homologous (*hsp16*; [11]) or heterologous (*Drosophila hsp70*; [12]) heat-shock promoters fused to a *lacZ* reporter gene, are inducible for β -galactosidase expression by a range of heavy metal and organic toxicants. Exposure temperatures about 3°C below the appropriate heat-shock induction threshold give optimal sensitivity to such exogenous stressors. Presumably these sub-heat-shock temperatures either potentiate or act in combination with the added stressor. Because the *C. elegans hsp16* gene is activated at 28°C [11] but the *hsp70* gene only at 34°C [8], it is advantageous to use the former strain for prolonged exposure proto-

cols, since this can be done at lower ambient temperatures, closer to the range routinely used for culturing *C. elegans* (15–25°C). Recent studies have confirmed that metal-polluted field samples can also induce transgene responses in these strains [13]. *C. elegans* has found some favour as a model organism for both aquatic [14] and soil-based [15] toxicity testing using the standard LC50 approach. Thus, stress-inducible transgenic *C. elegans* strains should afford an opportunity to investigate biological stress responses evoked by weak electromagnetic fields [2,16,17] or microwave radiation. With regard to the latter, there have been reports of stress responses to microwaves involving HSP induction, although these effects are attributed to thermal mechanisms (e.g., Ref. [18]). In this report, we demonstrate microwave-induced stress responses in transgenic *C. elegans* which are both reproducible and simple (also cheap!) to detect. Several features of the observed responses argue against a simple thermal effect, although very localised heating cannot be excluded as yet.

2. Materials and methods

C. elegans strain PC72 [11] was a generous gift from Professor E.P.M. Candido (Dept. of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada); this is the integrated *hsp16/lacZ* construct earlier described as strain PC72ubln5 [19]. All chemicals were obtained from Sigma (Poole, Dorset, UK).

2.1. Worm culture

Worms were grown for 4 days at 15°C on nematode growth medium [NGM] agar plates (either 9 cm Petri dishes or 25-well square multiwell plates) pre-seeded with a lawn of *E. coli* strain P90C (*lac*-operon deleted; [12]), then washed off the plates and filtered through a 5 µm mesh. This allows only the smallest size classes of worm (essentially L1 and L2 larvae; [20]) to pass through. Larvae washed through the mesh were pelleted by centrifugation (1 min at 10 000 × *g* at 4°C) and added to fresh NGM agar dishes pre-seeded as above. Under these conditions, L1/2 larvae mature synchronously to adulthood within 4–5 days at 15°C [20], and young adults

appear to be the stage most susceptible to external stressors [12]. Synchronised cultures of young adults were used throughout this study.

2.2. Microwave generator

The TEM cell was 34 cm long and of a square cross-section; it was tapered to a maximum cross-section of 24 × 24 cm at the centre and 1.5 × 1.5 cm at the ends. The inner septum was central and 27/32 of the total width, giving a 50 Ω impedance which matched the load and cables. Power was limited by the matched load to 500 mW (27 dBm).

2.3. Exposure of synchronous *C. elegans* cultures to microwave radiation

Cultures of young adults were transferred to a 25°C incubator containing the TEM cell, with the matched load external to the incubator. Three experiments are reported here.

(i) *Time course*. Worms were exposed for 0, 2, 4, 8 or 16 h at 750 MHz and 27 dBm.

(ii) *Multiwell plates*. Twenty-five-well plates containing identical aliquots of worms in each well were exposed for 2 h at 750 MHz and 27 dBm. Three successive runs were set up by filtering L1/L2 larvae from the same parental worm population and growing them up to adulthood (5 days at 15°C). After exposure, the worms from corresponding wells were pooled for assay purposes. This both increases the mass of worms available for assay from each well position, and also helps to average out inter-well variability.

(iii) *Frequency/power effects*. Worms were exposed for 16 h at 750 or 300 MHz at nominal power settings of 27, 24 or 21 dBm.

In experiments (i) and (iii) [but not (ii)], two dishes were exposed simultaneously, one resting on the waveguide plate and the other immediately above the first; both were located centrally within the TEM cell. Two sets of controls were included in each of the three experiments; one set was shielded with aluminum foil and placed in the same 25°C incubator but outside the TEM cell, while a baseline set was maintained at 15°C for the same period. After exposure, worms were washed off the dishes with K medium (53 mM NaCl, 32 mM KCl; [14]), allowed to settle twice at 4°C (leaving bacteria in the super-

nant), pelleted by centrifugation as above, and washed twice with ultrapure water. Pellets were frozen at -20°C after removal of supernatant.

2.4. MUG assay for β -galactosidase activity

Some 50–100 μl of acetone was added to each worm pellet and allowed to evaporate in an airstream at room temperature, thereby fixing and permeabilising the worms [9]. Sensitivity in detecting β -galactosidase activity was maximised by using a fluorogenic MUG substrate (4-methylumbelliferyl- β -D-galactopyranoside; 0.1 mg ml^{-1}), which is cleaved by the enzyme to a fluorescent product (4-methylumbelliferone; MU). After 30 min at 37°C , the reaction (total volume 200 μl) was stopped by adding 50 μl of trichloroacetic acid (TCA; 25% w/v), centrifuged, and MU production estimated in the supernatant using a Hoefer DynaQuant 200 fluorometer, which was calibrated using known (nM) concentrations of MU. The fluorometer manufacturer's protocols were followed exactly throughout the MUG assay procedure. Because of variation in the numbers of worms assayed, MU production was measured per unit total protein. After MU determination, the remaining worm + protein pellet was washed with 5% (w/v) TCA, digested with 30 μl of 10 M NaOH, and then subjected to a modified procedure of Lowry et al. [21] using 2% w/v NaHCO_3 (in place of 2% Na_2CO_3 in 0.1 M NaOH). The 10 M NaOH treatment digests proteins effectively, although worm cuticles remain largely intact. Lowry assays were therefore centrifuged prior to reading the supernatant optical density at 595 nm, in order to eliminate any confusion arising from cuticular debris. β -galactosidase activities are expressed throughout in units of pmoles MU per mg total protein per hour.

2.5. Statistical analysis

The significance of differences between data sets was assessed using Welch's *t*-test, which does not assume equal variances in the populations compared; two-tailed *P* values were calculated throughout, and the limit for statistical significance was set at 5% ($P \leq 0.05$). In experiment (iii) above, all data were normalised relative to the corresponding 25°C controls (arbitrarily taken as 100%) in order to make

comparisons between different wavelengths and power settings. In experiment (ii) using a 5×5 25-well array (oriented centrally with the first row of wells closest to the source), two-way nonparametric ANOVA was used to assess whether rows or columns of wells were significantly different from each other ([22], pp. 344–354); we are grateful to Dr. Francis Gilbert for suggesting this test.

3. Results

Using a standard frequency of 750 MHz at a nominal power setting of 27 dBm, varying periods of continuous microwave exposure (from 0 to 16 h) were compared with 25°C controls kept in the same incubator for the same period of time (time course shown in Fig. 1). Of the duplicate dishes exposed

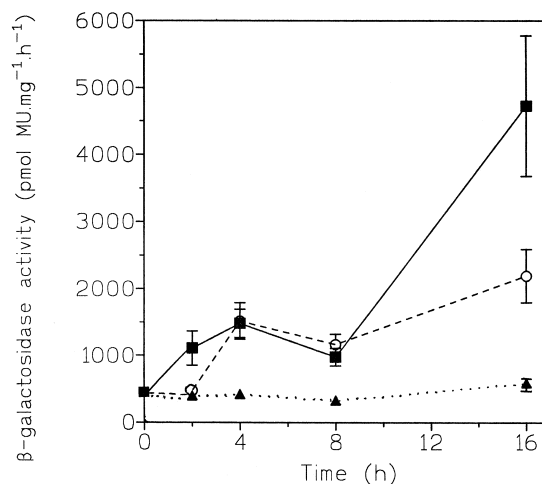


Fig. 1. Time course of stress response to continuous microwave radiation. Transgenic PC72 worms (synchronised young adults) were exposed to 750 MHz at 27 dBm and 25°C for varying periods of time (0–16 h), as described in Section 2. The 25°C controls were placed in the same incubator for the same periods, while baseline controls were similarly kept at 15°C . After exposure, worms were washed, fixed and assayed for β -galactosidase activity and total protein content as described in Section 2. Each point shows the mean of 8 replicates from 2 separate runs, together with the standard error (S.E.M.). Where error bars are not shown, they fall within the limits of the symbol used. Filled squares, solid line: enzyme activity following microwave exposure. Open circles, dashed line: enzyme activity in 25°C controls. Filled triangles, dotted line: baseline enzyme activity in 15°C controls.

within the TEM cell during each run, the lower dish (in contact with the metal waveguide plate) usually gave a higher response than the upper dish. This might suggest thermal heating effects with the waveguide plate acting as a heat conductor, although later results (see below) tend to argue against this possibility. However, since upper and lower dishes often gave responses that were higher than the corresponding 25°C controls, both upper and lower data sets have been pooled as 'exposed', and compared against the duplicate 25°C control dishes for each time point; the time course shown is pooled from several runs and its main features are reproducible. Microwave exposure resulted in significant induction of reporter-gene activity over 2 and 16 h (relative to 25°C controls), but paradoxically not over 4 or 8 h exposures. At 2 h, background expression of the reporter gene remained extremely low in the 25°C controls (no higher than at 15°C), whereas microwave exposure significantly increased the response ($P = 0.044$). By 4 h, however, the cumulative effect of low-level heat stress (only 3°C below the induction temperature for *hsp16*) resulted in reporter expression levels in the 25°C controls that were little different from those in microwave-exposed worms. Currently, we have no explanation for the slight drop in reporter gene expression observed in both exposed and control sets after 8 h (but this difference is not statistically significant). However, after 16 h the levels of induced reporter product were again significantly higher than in the corresponding 25°C controls ($P = 0.05$; Fig. 1). For this reason, all further work used either a 2 or a 16 h exposure. More detailed time course data will be needed to test the implication that controls respond to mild heat (25°C) with different kinetics from the response to microwave radiation.

In the multiwell plate experiment (Fig. 2), each of the 25 wells (5 × 5 square array) received an identical aliquot of L1 + L2 larvae (about 10^3). In order to assay larger numbers of worms without overcrowding and also to diminish inter-well variability, three sets of replica plates were set up on successive days (filtering larvae off from the same parent population each day), and each multiwell plate was tested after growing to adulthood for 5 days at 15°C (see Section 2). Worms washed out of corresponding wells were pooled from the three plates, which had all been

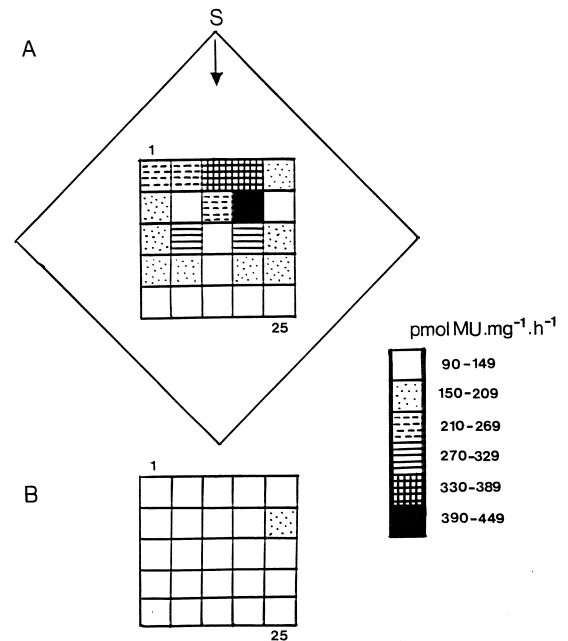


Fig. 2. Stress responses to microwave radiation in a multiwell plate. Identical aliquots of worms were cultured in each well of a 5 × 5 square multiwell array. Three replica exposures to 750 MHz at 27 dBm for 2 h were performed on 3 successive days (with the multiwell array in the same orientation and position each time), then worms were pooled from corresponding wells and assayed as above. The 25°C controls (as above) were similarly treated. The data are presented as a shaded scale map of response vs. position. The actual well size is 2 × 2 cm, separated by plastic walls 1.0 mm thick. The exposed 5 × 5 array was placed centrally within the TEM cell, resting on the waveguide plate, with the first row of wells (nos. 1–5) facing the source (Part A). The control array was placed in the same relative orientation (Part B), but was distant and shielded from the source. In both parts, the level of enzyme activity in each well position (pooled from 3 runs) is indicated by shading. Mean activities in A were significantly higher than controls (B); $P < 0.0001$. S = source.

placed in the same orientation during a 2 h exposure to microwave (750 MHz, 27 dBm; well numbers 1 to 5 closest to the source) or to control (25°C) conditions. Thus, the data in Fig. 2 are pooled rather than averaged from three similar experiments. Comparing enzyme activities in microwaved worms (196 ± 18 [S.E.M.] pmol MU mg⁻¹ h⁻¹; $n = 25$) with those in 25°C controls (95 ± 4.5 [S.E.M.] pmol MU mg⁻¹ h⁻¹; $n = 25$), the difference is highly significant ($P < 0.0001$). Fig. 2, however, reveals a striking clustering of high enzyme activities in certain regions of the 5 × 5 array, whereas medium and low

activities are more randomly distributed in controls. This presentation suggests that all of the high enzyme activities (plus a scattering of low activities, however) occur within the first three rows (wells 1–15) of the 5×5 array, i.e., in those wells closest to the source. By contrast, the last row (21–25) contains no high values at all and is indistinguishable from the control set. A nonparametric two-way ANOVA suggests that there are indeed significant differences between rows ($P < 0.05$), but not between columns ($P > 0.05$). In the 25°C control plates, there are no significant differences between either rows or columns ($P > 0.05$ for both); the distribution of activities does not deviate from random expectation (the null hypothesis). The fact that those rows closest to the source were more strongly affected than those furthest away strongly suggests some kind of shadow effect. These data also argue against any kind of local heating that could be conducted along the metal waveguide plate (on which the multiwell plate rested), since the last row of wells (21–25) was indistinguishable from control wells in terms of enzyme activity. It remains possible that power is absorbed locally by the agar medium (in effect an aqueous gel containing dissolved NaCl), and hence the row of wells closest to the source would absorb more power (causing greater local heating) than the more distant rows of wells. It is arguable whether such a temperature differential could be maintained over 2 h with only a thin (1 mm) plastic partition separating adjacent rows of wells. Direct temperature measurements (accurate to $\pm 0.1^\circ\text{C}$) undertaken in identical agar plates immediately after microwave exposure provide no evidence to support this suggestion (D. Thomas, unpublished observations). A temperature differential of at least 1.0°C would be needed to achieve a corresponding thermal increase in baseline β -galactosidase activity above 25°C controls (M. Mutwakil, unpublished observations).

The TEM cell used in this work performs optimally at 750 and 300 MHz, while the nominal power setting of 27 dBm was the maximum attainable with the matched load used. Further experiments were set up to test the effects of overnight (16 h) exposure to microwave radiation at both of the above wavelengths and also at power settings one half (24 dBm) and one quarter (21 dBm) of the maximum setting (27 dBm). In this set of experiments (Fig. 3), wide

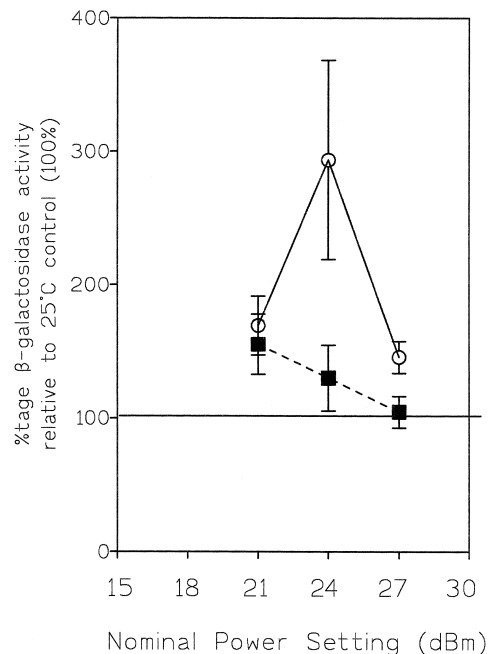


Fig. 3. Effect of varying power and frequency settings on the microwave response. Replicate Petri dishes (9 cm) were exposed overnight (16 h) to either 300 or 750 MHz at power settings of 27 (as in Figs 1 and 2), 24 or 21 dBm. The 25°C controls were set up as for Fig. 1. Because of variation in the 25°C control values following different exposure regimes (possibly reflecting temperature fluctuations in the incubator), each set of microwave-exposed dishes has been compared against its own set of 25°C controls (cf. for different time-points in Fig. 1), arbitrarily normalised to 100% (solid horizontal line). The variance in each control set has been taken into account in the statistical analysis (see Section 3). Each point represents the mean $\pm$ S.E.M. from 8 replicate assays in 2 separate runs. Open circles, solid line: response to microwaves at 750 MHz. Filled squares, dashed line: response to microwaves at 300 MHz.

variations were observed between different batches of 25°C controls. Because each set of control values represents the worms' response to ambient conditions within the incubator at the time of each exposure, it was appropriate to normalise each exposed data-set against the corresponding 25°C-control set, taking the mean of the latter arbitrarily as 100% throughout. Using this approach for the 750 MHz/27 dBm data-set (which corresponds to the 16 h time point in Fig. 1), the comparison between microwave-treated and 25°C-control worms is 146 ± 12 (S.E.M.) vs. $100 \pm 12\%$ ($P = 0.044$) in Fig. 3. The corresponding values from Fig. 1 are 215 ± 48

vs. $100 \pm 18\%$ ($P = 0.05$). These figures are broadly comparable, despite the scatter evident at 16 h in Fig. 1. At 300 MHz, the mean enzyme activities (relative to the 25°C set-controls as 100%) induced by microwave exposure are 105 ± 12 , 130 ± 25 and $155 \pm 23\%$ at 27, 24 and 21 dBm, respectively, of which only the last even approaches the threshold of significance ($P = 0.06$). At 750 MHz, the corresponding figures are 146 ± 12 , 294 ± 75 and $169 \pm 22\%$ at 27, 24 and 21 dBm, all of which are just significant relative to the corresponding 25°C control sets ($P = 0.044$, 0.041 and 0.050, respectively). The data for 24 dBm at 750 MHz show a large degree of scatter (hence the high S.E.M.), and should be treated with caution; it is unclear whether there is a real maximum effect here. At 300 MHz, there is an apparent (but not significant) upward trend in the effects observed as the nominal power setting decreases. More detailed investigation of the relationship between power setting (and/or frequency) and net induction above 25°C controls should prove instructive. At 750 and perhaps at 300 MHz, the maximum power setting tested (27 dBm) induces an apparently smaller transgene response than that induced at lower power settings. This observation tends to support our contention that power-dependent heating effects are unlikely to induce reporter expression in the test worms, although more clear-cut data would be needed to prove the point conclusively.

4. Discussion

4.1. Does microwave exposure cause significant biological effects?

Under some of the exposure conditions tested, reproducible and statistically significant induction of the reporter gene product (β -galactosidase) has been observed. For instance, taking the corresponding 25°C control as 100%, a 2 h exposure to 750 MHz at 27 dBm induces enzyme activity to $236 \pm 55\%$ (S.E.M.) in Fig. 1 ($n = 8$; $P = 0.044$), whereas the corresponding mean value for all wells in Fig. 2 is $207 \pm 19\%$ ($n = 25$; $P < 0.0001$). There is also fair agreement between the effects of 16 h exposure to the same microwave conditions between Figs. 1 and 3. By using multiple replicates (either on the same or

different occasions), it should be possible to detect smaller responses under less severe microwave exposure conditions (different wavelengths and/or altered power settings and/or shorter exposure times). *C. elegans* is unusual in that a large temperature shift is needed to induce its heat shock genes [23]; although routinely cultured at 20°C, the *hsp16* gene is activated only at 28°C and the *hsp70* genes only above 33°C [23,8,11,12]. This may reflect the worm's innate tolerance of adverse conditions, itself a feature of its opportunistic lifestyle [24]. In fact, *C. elegans* populations can survive and reproduce very well at temperatures as high as 25°C [10], which was the control temperature used in these studies. This is only 3°C below the induction temperature for the *hsp16* promoter which regulates reporter gene expression in our transgenic strain, PC72. Arguably, this is a more realistic approximation to the human situation, where a 4°C rise in ambient temperature (from 37 to 41°C) is sufficient to initiate a heat-shock response. The levels of response observed in worms exposed to microwaves are comparable to the effects of metals such as Cu^{2+} , Zn^{2+} and Mn^{2+} at concentrations between 2 and 10 ppm [12], using an 8 h exposure at 25°C for strain PC72. Prolonged exposure of *C. elegans* to ppm concentrations of these metals is lethal after 24–48 h [14], and certainly such concentrations are regarded as injurious to human health. Although the transgenic worm assay described here is simple, cheap and convenient, it is not particularly sensitive to exogenous stressors (for the reasons described above). It is likely that *hsp*/reporter constructs carried in transfected vertebrate cell-cultures (e.g., Ref. [25]) will prove much more sensitive for biomonitoring microwave- or EMF-induced stress. This might allow the detection of measurable stress effects at much lower frequencies and/or power levels.

4.2. Are the observed effects due to heating?

Since higher frequency microwaves (2.45 GHz, a resonant frequency of H_2O) are used at much higher power settings (0.5–2.5 kW) in microwave ovens, heat generation would afford an obvious mechanism for inducing a heat shock response. Indeed, other workers have used microwave-induced heat stress as an experimental tool [18]. Given that the *hsp16*

promoter becomes maximally active at 28°C, a twofold induction above 25°C controls would require a temperature rise of approximately 1.0°C; this estimate is based on temperature response curves for strain PC72 (M.H.A.Z. Mutwakil, unpublished data from this laboratory). Given that power might be absorbed locally, much would depend on conduction and convection effects within the agar gel, on which the worms crawl. These effects are difficult to quantify, and localised heating effects cannot as yet be ruled out rigorously. Generalised heating affecting the entire dish appears much less plausible. In Fig. 1, the data suggest different kinetics for the microwave response as compared to the effects of moderate heat alone (25°C controls). Because dishes in contact with the metal waveguide plate showed stronger responses than replica dishes placed above them (with an insulating layer of air between the agar surface of the lower dish and its lid supporting the upper dish), it seemed possible that heat was being conducted through the waveguide plate. However, this explanation was ruled out by the multiwell data shown in Fig. 2, where maximal effects were observed in the well-rows closest to the power source, while the most distant row was comparable to 25°C controls. This could be explained in terms of power absorption by the agar gel, creating a kind of shadow effect further from the source. Thus, it remains possible that localised heating effects directly induced by microwaves could explain the observed stress responses. Finally, reducing the nominal power settings to one half or one quarter of the initial setting did not markedly diminish the induced response (Fig. 3). Heating effects would be expected to increase in line with the power setting, but our observations (over six test conditions) fail to confirm such a trend. It is possible that microwave radiation might interact with biological structures (causing protein damage) by mechanisms that do not directly involve heat; one key factor here might be the resonant frequencies of the weak bonds that maintain proteins in their biologically active 3-dimensional configuration (since disruption of this would lead to protein denaturation and hence *hsp*-gene activation). This might, for example, account for the induction of similar stress responses in cells exposed to low-frequency (60 Hz) electromagnetic fields [16,17]. However, the variable responses observed to 750 MHz microwave exposure

at 24 dBm (Fig. 3) raise the very problem of reproducibility which has plagued many other studies in this field (reviewed in Ref. [1]). Examination of the raw data suggests that, under the same exposure conditions, one batch of worms responded very strongly, but the other hardly at all (both of these effects were consistent within a group of replicates). Worms from these same batches showed more uniform responses to 750 MHz microwave radiation at both 27 and 21 dBm (Fig. 3), in spite of considerable inter-batch variation among the 25°C controls (normalised as 100% throughout). It may be that 24 dBm approximates to a narrow power-window for response, or that other factors such as crowding or precise developmental stage could influence the response. Clearly, further work will be required to illuminate this confusing point. On the other hand, it should be noted that this is the only data point in our study for which such large disparities exist.

4.3. Future directions

One important avenue of current research is to explore the effects of pulsed signals on the stress responses observed in transgenic worms. Preliminary evidence suggests that long pulses (15 min or 1 h) separated by equal or longer intervals (15, 45 min, or 2 h) have relatively little effect (less than continuous exposure), implying that the worms can recover from microwave-induced stress during the inter-pulse interval. By contrast, with much shorter pulses (40 s, separated by 40 s intervals), the overall response appears to be comparable or enhanced as against a similar period of continuous exposure (C. Daniells and D. de Pomerai, unpublished results). If confirmed, this might suggest that a damage threshold is being exceeded repeatedly without sufficient time for recovery between pulses, so building up a stronger cumulative response. Further exploration of this question may have important implications for advising on the safe use of mobile phones and other devices operating in the microwave frequency range. Typical frequencies used in mobile phone equipment are 900 and 1800 MHz, with a power output of around 0.6 W (28 dBm). These conditions are not dissimilar to our standard exposures at 750 MHz/27 dBm, which form the basis of Figs. 1 and 2. The transgenic nematode system could also be used to

examine the biological effects of low-frequency electromagnetic fields, which have been reported to induce *hsp* gene expression [2,16,17]. Although nematode-based assays will be useful for focusing attention on those microwave/EMF exposure conditions that can cause biological damage (with consequent *hsp*-gene induction), it is likely that transfected cultured cells [25] will prove more sensitive and informative as regards the mechanisms of damage. With the advent of novel transgenic strains of *C. elegans* carrying Green Fluorescent Protein reporter constructs (whose expression can be monitored *in vivo*), it would be possible to separate responder from nonresponder worms following microwave or EMF exposure, with a view to comparing long-term survival, reproduction and mutation rates among their progeny. Given its short generation time (3–4 days at 20°C), *C. elegans* is an ideal organism for such studies.

Note added in proof. Since acceptance of this article, we have further investigated the possibility that the observed stress responses might be caused by heating. Temperature measurements in all 25 agar-filled wells of a multiwell plate immediately following removal from the TEM cell after 2 h of standard microwave exposure (750 MHz/27 dBm) gave a mean of 24.7°C as compared to 24.75°C for shielded controls in the same incubator. Continuous temperature measurements with a nonmetallic probe resting on the agar surface within the TEM cell showed no inflection when the signal generator was switched on, and no detectable rise in temperature over a 2 h exposure period. Both of these approaches would have detected temperature differences of 0.2°C or less. Since temperatures in excess of 26.5°C would be needed for comparable (twofold) induction of transgene activity above 25°C controls, we believe that the generalised heating hypothesis can be discounted.

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