Title.

A small temperature rise may contribute towards the apparent induction by microwaves of heat-shock gene expression in the nematode *Caenorhabditis elegans*.

Authors.

Adam S. Dawe*, Brette Smith*, David W.P. Thomas#, Steve Greedy#, Nebojsa Vasic#, Andrew Gregory**, Benjamin Loader** and David I. de Pomerai*[1].

Affiliations.

* Institute of Genetics, School of Biology and #George Green Institute of Electromagnetic Research, School of Electrical and Electronic Engineering, both at the University of Nottingham, Nottingham NG7 2RD, United Kingdom.

**Enabling Metrology Laboratory, National Physical Laboratory, Queens Road, Teddington TW11 0LW, United Kingdom.

[1]Corresponding Author:

Telephone: 44 115 951 3250. Fax: 44 115 951 3251.

e-mail: david.depomerai@nottingham.ac.uk

Running Title:

Thermal contribution towards heat-shock induction by RF.

Funding:

This study was funded by the UK Mobile Telecommunications Health Research (MTHR) initiative, and in part by the Fund for Children with Leukaemia (FCL, funding a PhD studentship to BS).

Abstract:

We have previously reported that low-intensity microwave exposure (0.75-1.0 GHz CW at 0.5 W; SAR 4-40 mW kg^{-1}) can induce an apparently non-thermal heat-shock response in *Caenorhabditis elegans* worms carrying *hsp16-1*::reporter genes. Using matched copper TEM cells for both sham and exposed groups, we can detect only modest reporter induction in the latter (15-20% after 2.5 h at 26°C, rising to ~50% after 20 h). Traceable calibration of our copper TEM cell by the National Physical Laboratory (NPL) reveals significant power loss within the cell (8.5% at 1.0 GHz), accompanied by slight heating of exposed samples (~0.3°C at 1.0 W). Thus exposed samples are in fact slightly warmer (by ≤0.2°C at 0.5 W) than sham controls. Following NPL recommendations, our TEM cell design was modified with the aim of reducing both power loss and consequent heating. In the modified silver-plated cell, power loss is only 1.5% at 1.0 GHz, and sample warming is reduced to ~ 0.15°C at 1.0 W (i.e. ≤ 0.1°C at 0.5 W). Under sham:sham conditions, there is no difference in reporter expression between the modified silver-plated TEM cell and an unmodified copper cell. However, worms exposed to microwaves (1.0 GHz and 0.5 W) in the silver-plated cell also show no detectable induction of reporter expression relative to sham controls in the copper cell. Thus the 20% "microwave induction" observed using two copper cells may be caused by a small temperature difference between sham and exposed conditions. In worms incubated for 2.5 h at 26.0, 26.2 and 27.0°C (with no microwave field), there is a consistent and significant increase in reporter expression between 26.0 and 26.2°C (by ~20% in each of 6 independent runs), but paradoxically expression levels at 27.0°C are similar to those seen at 26.0°C. This surprising result is in line with other evidence pointing towards complex regulation of *hsp16-1* gene expression across the sub-heat-shock range of 25-27.5°C in *C. elegans*. We conclude that our original interpretation of a non-thermal effect of microwaves cannot be sustained; at least part of the explanation appears to be thermal.

Key Words:

Reporter transgene / radio-frequency fields / thermal versus non-thermal effects / stress response

Introduction:

We have previously reported that low-intensity microwave fields (continuous wave, at 750 MHz or 1.0 GHz; 0.5 W power input) can elicit a clear heat-shock response in the model nematode *Caenorhabditis elegans* (Daniells *et al.*, 1998; de Pomerai *et al.*, 2000a, b). This was detected using transgenic strains carrying stress-inducible *hsp16-1*::reporter constructs (principally a *lacZ* reporter in strain PC72), in which heat-induced expression becomes apparent only at temperatures exceeding 27°C (de Pomerai *et al.*, 2000a). At exposure temperatures of 25-26°C, PC72 worms are sensitive to a variety of exogenous stressors, including heavy metals, pesticides, anti-worm-surface antibodies, and microwaves (Power *et al.*, 1998). In all cases, exposure to the test stressor for between 2 and 24 h results in a marked induction of reporter-gene expression (as β-galactosidase and/or GFP), relative to non-exposed control worms derived from the same source population. Inter-run consistency is improved by using synchronised worm cultures at the same developmental stage (usually L4 larvae), and by controlling worm numbers, aeration and the availability of bacterial food. Because of their small size (~1 mm), the responses of 10^2-10^3 worms are averaged within each sample, and the simplicity of worm culture allows multiple replicates of many different test conditions to be assayed simultaneously. Recent data indicates that fluorescence signals from GFP reporters (e.g. in the *hsp16-1*::GFP::*lacZ* double-reporter strain PC161; David *et al.*, 2003) can be measured from as few as 100 worms per well in a 96-well microplate format suitable for high-throughput screening. These features allow extensive replication under near-identical test conditions, which should allow even small responses to be picked up unambiguously.

The heat-shock response is properly described as a general cellular stress response, since production of the characteristic heat-shock proteins (HSPs) is induced by a wide variety of environmental insults (Sanders, 1993). Most HSPs function as molecular chaperones, binding to unfolded proteins and assisting them to refold correctly after stress-induced conformational damage (Parsell and Lindquist, 1993). Small HSPs (including *C. elegans* HSP16s) assemble into large multimeric complexes, which act to prevent aggregation of partially unfolded protein chains (Leroux *et al.*, 1997). Stress-inducible HSP genes are activated by the heat-shock transcription factor, HSF (Lis and Wu, 1993), which undergoes a multi-stage activation process before binding to heat-shock elements (HSEs) in the target HSP gene promoters and stimulating transcription. In *C. elegans*, the *hsp16* genes are induced by HSF at lower

temperatures (>28°C) than other inducible HSP genes such as *hsp70i* (>33°C; Snutch and Baillie, 1983). Worms grow normally up to 25°C, thus HSP16 seems to provide a front-line defence against mild stress.

The induction of *hsp16-1*::reporter expression by microwaves is strongly temperature-dependent, since this response is undetectable at 24°C but increases steeply from 24.5 through 25.0 to 25.5°C (de Pomerai *et al.*, 2000a). Although there is a plateau of low-level expression at and above 25°C in shielded controls, reporter gene activity does not increase steeply until the temperature exceeds 27°C. This apparent shift of 2-3°C in the temperature profile for *hsp16-1*::reporter induction led us to propose that the underlying mechanism might be non-thermal (de Pomerai *et al.*, 2000a). This suggestion drew apparent support from the estimated SAR range of 4-40 mW kg^{-1} (based on both TLM- and FDTD-based modelling approaches, and confirmed by NPL field measurements) – which is far too low to cause direct microwave heating – and also from temperature measurements with a microthermocouple (de Pomerai *et al*, 2000a), showing that exposed samples are only around 0.1°C warmer than shielded controls immediately after exposure.

Our recent work uses two identical copper TEM cells placed on the same incubator shelf, housing the exposed (1.0 GHz CW, 0.5 W) and sham groups, respectively. Most experiments also use a reduced exposure time of 2.5 h, so as to minimise confounding effects from starvation and anoxia. This protocol gives far less dramatic induction of reporter expression by microwaves than that seen in previous reports, although it is still significantly higher than in sham controls (by ~20% over 2.5 h). We show below that power loss within the live copper cell results in slight heating (exposed samples are ≤0.2°C warmer than sham controls at 0.5 W), but when this disparity is reduced by ~50% after modifying the TEM cell, there is no longer any induction of reporter expression following microwave exposure. Moreover, a small temperature rise of ~0.2°C can activate reporter expression to a similar extent (~20%) to that seen during microwave exposure in matched copper cells; paradoxically, a larger rise of 1°C does not increase this effect (reporter expression at 27.0°C is similar to that at 26.0°C, yet both are less than at 26.2°C). Taken together, our findings suggest that slight heating is sufficient to explain the modest effects attributed to microwave exposure using matched copper cells. However, they do not entirely account for the much larger effects reported previously, which we have never been able to reproduce using matched sham and exposed cells. Other differences between shielded and exposed conditions may contribute to this contrast.

Materials and Methods:

Materials: *C. elegans* strain PC72 was generously donated by Dr E.P.M. Candido (Department of Biochemistry and Moecular Biology, University of British Columbia, Vancouver, Canada), while the *lac*-operon-deleted P90C strain of *E. coli* was originally from Dr A. Chisholm (MRC Laboratory of Molecular Biology, Cambridge, UK). PC161 worms were engineered in Dr Candido's laboratory by Dr H. David (David *et al.*, 2003). All chemicals were Ultrapure grade from Sigma, and plastic disposables were from Nunc Ltd. unless otherwise stated.

Calibration: The TEM cell used here was identical to that described by Daniells *et al.* (1998), except that it was constructed of copper rather than aluminium. It was 34 cm long and of a square cross-section, tapering from a maximum of 24 x 24 cm at the center to 1.5 x 1.5 cm at the ends. The inner septum (waveguide) 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). The S-parameters of this TEM cell were measured at NPL (B Loader and A Gregory) with a calibrated network analyzer. Measurements were made both with and without the presence of a loaded 24-well multiwell plate, containing 1.0 ml per well of K medium (53 mM NaCl, 0.32 mM KCl). Temperature rises corresponding to 1.0 W and 10 W power inputs (into a 50 Ω load) were measured using a T1V3 temperature probe (Schmid & Partner Engineering, Zurich), which is designed for temperature measurements in a high field environment. The outer probe diameter is 1 mm, with an absolute accuracy of 10 mK and a measurement noise of 0.5 mK RMS over a 10 sec measurement time. All temperature measurements were conducted with the probe dipping into the liquid K medium in a loaded 24-well plate (as above). Following modifications to the TEM cell recommended by NPL (including removal of internal polystyrene foam from beneath the septum, replacement of BNC by APC 3.5 connectors, and silver plating the copper surfaces of the cell), the frequency response and temperature measurements were repeated. Note that one set of temperature measurements was conducted at 21°C, but the other at 19°C; these were simply the ambient laboratory temperatures at the National Physical Laboratory (AG and BL) on the measurement dates in question. As an additional check, these temperature measurements have been repeated *in situ* at Nottingham, using the same probe for 1 ml K medium samples in both the copper and silver-plated TEM cells inside a 26°C incubator routinely used to house both sham and exposed cells. These measurements

confirm both the temperature rises previously calibrated in an open laboratory environment at NPL, and also the ~2-fold difference in sample heating between these cells.

Worm culture, exposure and assay: PC72 worms were grown on NGM agar plates as described previously (Sulston and Hodgkin, 1988), and were synchronized by L1 filtration (Mutwakil *et al.*, 1997) or by egg isolation using NaOCl (Sulston and Hodgkin, 1988). 1000 synchronous L4 larvae were placed in 1.0 ml of K medium into each well of a 24-well microplate kept on ice. Identical plates were placed in two copper TEM cells located on the same shelf of a large incubator set at 26.0 (± 0.1)°C; one of these was exposed to an RF field (1.0 GHz continuous-wave signal; 0.5 W power input) while the other was sham-exposed in the second cell with no RF field applied. After exposure for 2.5 h at 26°C, plates were immediately frozen for later reporter analysis. On thawing, worms were carefully resuspended and pelleted by centifugation (4,000 x g for 1 min at 4°C), then subjected to a standardised MUG assay (as described in David *et al.*, 2003) to detect induced β-galactosidase reporter activity. This enzyme converts the substrate 4-methylumbelliferyl-βD-galactopyranoside [MUG] into 4-methylumbelliferone [MU], which is strongly fluorescent in alkaline solution. Each pair of column bars shown represents a single run (exposed or sham versus sham), and gives the mean ± SEM derived from all 24 replica wells within each plate. In later experiments, a modified silver-plated TEM cell was used for exposures, and an unmodified copper TEM cell was used for shams. All MUG assay results are given in pmol MU h^{-1} ml^{-1}. For the PC161 double-reporter strain (Figure 1B only), exposed and sham groups were placed in non-fluorescent black multi-well plates at 26°C (David *et al*, 2003), and test treatments were continued for a total of 20 h. At 2- or 4-hourly intervals each plate in turn was removed and GFP fluorescence measured in all wells using a Perkin-Elmer Victor 1420 multi-label plate reader (with FITC filters for optimal GFP detection); this procedure took < 5 min in total for each plate, after which it was returned immediately to the appropriate test condition. Thus the time-course in Figure 1A shows sequential measurements of the **same** sample sets at successive time-points. In this case, all GFP fluorescence measurements were normalised relative to the initial value at t = 0 (as 100%). In all of the above experiments, negative and positive heat-shock controls were also run at 15 and 30°C, respectively. The results of these controls are not presented here, but for the *lacZ* reporter in PC72, the intensity of expression was 30 >> 26 ≥ 15°C at all time points. For PC161, this order was similar, apart from a lag seen only at 30°C (David *et al*, 2003).

Effects of temperature alone on heat-shock reporter expression: PC72 L4 worms (as above) were distributed among several 24-well plates and incubated for 2.5 hours in incubators previously set to 15.0, 26.0, 26.2 and 27.0°C (also at 30°C as above; data not shown). Temperatures were checked with maximum/minimum and narrow-range mercury thermometers as well as TinyTalk temperature loggers and Fluke 54 II digital thermometers across the key 26.0-27.0°C range; all these concurred on the stated temperatures to within ± 0.1°C. Temperature monitoring equipment was moved around among the three incubators during six successive runs (seven for 26.0 versus 27.0°C), in order to reduce the effects of any consistent errors between different thermometric devices. Because none of the instrumentation used is accurate beyond the first decimal place (i.e. ± 0.1°C), we cannot claim greater accuracy in terms of the actual temperatures experienced by worms. In some runs, incubator temperatures were reset (usually over the preceding weekend), so that the same target temperatures were achieved using a different combination of incubators. This did not affect the differing levels of reporter expression observed. Overall, despite calibration inaccuracies, the order of temperatures is clearly 27.0 >> 26.2 > 26.0°C. The results of this experiment were normalized relative to the mean reporter expression measured at 15.0°C in each run (as 100%), and statistical comparisons were made between results at 26.0, 26.2 and 27.0°C.

Results:

~~**Results:**~~

A small octahedral TEM cell was designed and built at Nottingham some years ago (by DWP Thomas), and those used here differ only in respect of their construction material (copper rather than aluminium) from the version used in our earlier studies (Daniells *et al.*, 1998; de Pomerai *et al.*, 2000a, b). The live cell is connected to a Laplace RF1000 signal generator at the input and to a matched 0.5 W load at the output. Worms are normally held in 24-well multiwell plates containing 0.5-1.0 ml of saline (K medium) per well, placed centrally on the wave-guide septum. Sham controls are housed in an identical TEM cell placed on the same incubator shelf; in sham:sham runs, temperature sensors placed in both TEM cells give essentially indistinguishable and very stable temperature profiles (26.0 ± 0.1°C over 20 h).

Using two matched copper TEM cells, a 2.5 hour exposure to microwaves at 26°C routinely results in a 15-20% induction of *hsp16-1*:: reporter gene activity (β-galactosidase for the PC72 worms used here), as shown for five independent experiments in Figure 1A. Differences between exposed and sham-control mean activities are significant ($p < 0.05$,) both within each run and across the 5 runs (normalising data relative to shams in each case). However, this level of induction is far lower than the 4- to 5-fold differences between exposed and shielded conditions reported previously (de Pomerai *et al*, 2000a), although this will result in part from the shorter exposure times used here (2.5 h rather than 20 h). Figure 1B shows a 20 h time-course for microwave-exposed versus sham PC161 worms (David *et al*, 2003); this strain was chosen because GFP fluorescence can be monitored at intervals in the same groups of worms, allowing the responses to be monitored dynamically. After 2 h, GFP expression is already higher in exposed (112% relative to t = 0) as compared to sham worms (97%), consistent with the differences seen for PC72 worms in Figure 1A. By 20 h, this difference has widened considerably, to 150% in exposed versus 110% in sham. Apparent differences between control (sham or shielded) and exposed conditions will be influenced by the choice of baseline for normalisation (here t=0), a point discussed further below.

Traceable calibration of our copper TEM cell at the UK National Physical Laboratory (NPL) revealed significant power loss within the cell, far greater than the energy deposited in exposed samples by the microwave field. As shown in Figure 2, as much as 8.5% (± 0.9%) of the input power is lost at 1.0 GHz, and presumably much of this will be dissipated as heat within the cell (see below and Figure 3). Our

NPL colleagues (AG and BL) therefore recommended several changes to the design of our TEM cell, with the aim of minimising this power loss problem. The principal modifications were as follows:-

(i) Removing internal packing (expanded polystyrene) from beneath septum.

(ii) Supporting septum instead with small equatorial polystyrene pegs.

(iii) Lengthening all cabling and replacing BNC with APC 3.5 connectors.

(iv) Silver-plating all internal surfaces (in fact, the whole cell was sputter-coated with silver).

These changes were duly effected and the modified cell (henceforth termed the Ag cell, as distinct from the unmodified Cu cell) was subjected to the same calibration regime. As shown in the lower trace of Figure 2, power loss has been reduced by > 4-fold as a result of these modifications, since less than 1.5% (± 0.2%) of input power is lost at 1.0 GHz in the Ag cell.

Temperature measurements were carried out during sample exposure (1 ml K medium per well in a 24-well plate) for both the Cu and Ag cells, as shown in Figure 3. These two sets of measurements were carried out on separate occasions at different ambient laboratory temperatures – 19°C for the Cu cell and 21°C for the Ag cell (see Methods). For the unmodified Cu cell, sample temperatures were increased by ~2.6°C using a 10 W power input (Figure 3A) and by 0.26°C at 1.0 W power input (Figure 3C). Since heat dissipation should be greater the further the temperature is raised above ambient, one would expect the ratio of the resultant temperature rises to be somewhat less than the 10-fold ratio of power inputs. Since the data in Figure 3 show these ratios to be ~10-fold for the temperature rises in both cells at 10 W versus 1.0 W, we must conclude that the temperature probe used may underestimate small temperature rises. For this reason, the rise observed at 1.0 W in the copper cell is henceforth described as ~0.3°C; on this basis, we predict a temperature rise of ≤ 0.2°C in samples exposed under standard conditions with a power input of 0.5 W. For the modified Ag cell, sample temperatures were increased by 1.3°C at 10 W (Figure 3B) and by 0.13°C (henceforth ~0.15°C) at 1.0 W power input (Figure 3D); at 0.5 W we would

anticipate a temperature rise of ≤ 0.1°C. The temperature rise (power on) and fall (power off) are in all cases steep initially, but a plateau temperature equilibrium is established within the first hour of exposure and no further temperature rise can be detected thereafter. Although the power loss problem has been largely solved by the modifications made in the Ag cell, these have only partially solved the problem of sample heating, reducing this from ≤ 0.2°C during exposure in the Cu cell to ≤ 0.1°C in the Ag cell. Dawe, p. 10.

In view of the heating identified above (Figure 3), a question arises as to whether the modest stress-responses seen in Figures 1A and B might be explicable in terms of a small temperature rise (≤ 0.2°C) experienced by the exposed group relative to sham controls in the copper TEM cells. To this end, PC72 worms were exposed under identical run conditions in the modified Ag cell. Because only one of our TEM cells was modified, we first compared the Ag and Cu cells under sham:sham conditions. As shown in Figure 4A, in nine 2.5 h replica runs we could find no evidence for any consistent difference in reporter expression between these two cells (the few small differences are non-significant and inconsistent). We then conducted standard microwave exposures in the Ag cell (where accompanying heating is ≤ 0.1°C), as compared to shams in the Cu cell. As shown in Figure 4B, across ten 2.5 h replica runs we could find no net induction of reporter activity by microwaves, even though the fields applied are essentially the same as in Figure 1A (a point verified by SAR measurements on both cells at NPL and at Nottingham; data not shown). The consistency of expression levels across all 10 runs only serves to reinforce this negative conclusion. Reducing the temperature differential between exposed and sham samples (from ≤ 0.2°C in Figure 4A ≤ 0.1°C in 4B) has effectively abolished any detectable stress response to microwaves.

We therefore looked more closely at the response of *hsp16-1* reporter expression to small increases in temperature. Figure 4C shows the effect of incubating PC72 worms at temperatures of 26.0, 26.2 and 27.0°C relative to 15°C (as explained in Methods, temperature measurement accuracy was ± 0.1°C). Expression levels are substantially increased at 26.2°C (126 ± 2.5% SEM, relative to 100% at 15°C; n = 6 runs) as compared to 26.0°C (106.7 ± 3.1% SEM; n = 6), a difference which is statistically significant ($p < 0.01$, using 1-way ANOVA and a Tukey-Kramer *post hoc* multiple comparisons test). Surprisingly, however, a further temperature increase up to 27.0°C does not increase reporter expression levels (109.3

± 3.6% SEM, n = 6) above those seen at 26.0°C (difference between 26.0 and 27.0°C not significant, $p > 0.05$). Counter-intuitively, in each of the six runs performed, expression levels at 26.2°C are significantly higher than those at 27.0°C ($p < 0.01$). The implications of this are further discussed below.

Discussion:

Using two matched copper TEM cells for sham and microwave exposures, we have never been able to reproduce the combination of high induction and very low background (control) expression seen in earlier studies using a single aluminium exposure cell and shielded controls (Daniells *et al*, 1998; de Pomerai *et al*, 2000a, b). Clearly, sham controls are more closely matched to the exposure conditions than are shielded controls in a foil-wrapped box placed outside the TEM cell but at the same level within the incubator; thus extraneous differences between shielded and exposed conditions may compound the effects described previously. Nevertheless, a smaller (~20%) but reproducible effect of microwave exposure can be detected after 2.5 h using the matched copper cells (Figure 1A), rising to ~50% after 20 h (Figure 1B). However, all such comparisons will be greatly affected by the starting point used for normalisation. For the time courses in Figure 1B, time zero values (for the whole source population) provide a logical basis for such normalisation. In our earlier studies, expression levels were normalised against controls held at 15°C – where basal expression is normally slightly lower than for time zero at 26°C. If, for the sake of argument, this 15°C baseline were at ~80% of the t = 0 baseline, then the 20 h expression levels in Figure 1B would increase to ~80% (nearing 2-fold) induction in exposed versus ~30% in sham controls. To avoid this dependence on normalisation against an arbitrarily chosen baseline, raw expression data are presented for all sham:exposed comparisons in Figures 1A, 4A and 4B. The slopes of the sham and exposed curves will also affect the outcome in terms of net apparent induction. Within the same experiment shown in Figure 1B, simply doubling the sample volume results in a steeper increase in reporter expression under sham conditions (data not shown), probably due to the worms becoming anoxic and stressed after many hours at a greater depth below the air:medium interface.

The question of whether microwave radiation can affect biological systems by mechanisms not involving heating has been hotly debated. There is currently no recognised biophysical basis for such non-thermal

effects (Adair, 2002), although there have been suggestions that microwave fields might alter the rates of protein folding and unfolding (Bohr and Bohr, 2000; de Pomerai *et al.*, 2003). This mechanism could plausibly explain the induction of a modest heat-shock response (de Pomerai *et al.* 2000a, b; Leszczynski *et al.*, 2002) via accumulation of partially unfolded proteins within cells. However, these effects on protein structure are only thought likely at higher power levels and higher frequencies than those used in

our work. The results presented in this paper suggest that small temperature changes may affect heat-shock reporter expression to a greater extent than previously suspected. To summarise, a modest "microwave response" is apparent as a 20% increase in *hsp16-1*::-reporter expression when the temperature of exposed samples exceeds that of sham controls by ≤ 0.2°C in the original Cu cell (Figures 1A and 3C), but this induction is abolished in the modified Ag cell where the temperature differential has been halved to ≤ 0.1°C (Figures 4A and 3D). Given that the microwave field within the TEM cell is essentially unchanged (as confirmed using a network analyser and SAR measurements) and that all protocols were identical between these runs, there remains only a small temperature difference (≤ 0.1°C) to differentiate conditions inside the Cu and Ag cells during microwave exposure.

Air temperatures inside our fan-assisted incubators show rapid oscillations (range 0.2-0.5°C) due to their thermostatic controls, but these are dampened inside the Ag cell and completely smoothed inside the unmodified Cu cell (thanks to the insulating effect of the polystyrene packing under the septum). However, these oscillations do not affect liquid K-medium samples, which rapidly (within 20 min) equilibrate to the set incubator temperature in all exposure cells. Sham controls inside a TEM cell (this paper) are ~0.1°C warmer than foil-wrapped shielded controls placed at the same level in the incubator (as used in our earlier work; de Pomerai *et* al., 2000a, b). Added to the known heating caused by power losses within our TEM cell (Figures 2 and 3), this suggests that exposed samples could be as much as 0.3°C warmer than shielded controls in our earlier work, and ≤ 0.2°C warmer than sham controls in a matched TEM cell (Figure 3C). Rapid cooling of samples after switching off the power (Figure 3) may perhaps explain why previous temperature measurements using a microthermocouple immediately after exposure could only pick up differences of around 0.1°C (de Pomerai *et al*, 2000a). Even so, could such small temperature differences (≤ 0.2°C) suffice to account for modest the modest heat-shock responses seen in Figures 1A and B, or should these be attributed to some non-thermal effect of RF exposure?

Figure 4C suggests that the thermal explanation is probably sufficient, although it is difficult to control incubator temperatures to an accuracy of better than ± 0.1°C. We tried alternative strategies, including a DC offset device to heat the TEM cell septum (where heat was largely dissipated through the load) and hybridisation ovens (whose temperature control is accurate only at higher ranges), but without success.

Temperature calibrations for Figure 4C used a variety of measuring devices, which were rotated around different combinations of incubators between the 6 runs. A small temperature rise of ~0.2°C (from 26.0 to 26.2°C) can significantly increase *hsp16-1*::reporter expression (by ~20% relative to 15°C; $p < 0.01$). This suggests that the comparable inductions (~20%) seen in Figure 1A could be explained thermally, and do not require any additional contribution from microwave radiation. But does this further imply that larger temperature rises would induce correspondingly stronger responses in terms of reporter expression? The data in Figure 4C paradoxically suggest otherwise; since levels of reporter expression at 27.0°C are very similar to those at 26.0°C, and are in all cases below those seen at 26.2°C (this holds true for all 6 runs shown in Figure 4C). We do not wish to overstate the significance of this surprising result without further corroboration. Taken at face value, the evidence presented in Figure 4C would at least suggest the possibility of thermal "window effects" which, if confirmed, could further confound many alleged effects of electromagnetic fields on biological systems. The similarity of *hsp16-1* expression levels at 26.0 and 27.0°C (this paper) might also help to explain an apparent plateau of low-level reporter expression seen in shielded controls across a temperature range from 24.5 to 27.0°C (de Pomerai *et al*, 2000a). Whether or not there might be cyclical fluctuations in *hsp16-1*expression across this range must remain a topic for speculation, since to our knowledge this question has never been investigated in detail.

Independent evidence also indicates that thermal control of small *hsp16* heat-shock genes in *C. elegans* by HSF is more complex than previously thought. HSF turns out to be an important co-regulator (along with DAF-16) of many stress- and ageing-related genes (Hsu *et al*., 2003) during normal development, as well as under stress. Although HSF activates only the small *hsp16* heat-shock genes under mild heat-stress conditions (28-32°C), at higher temperatures these are down-regulated and the inducible *hsp70i* genes activated instead (> 33°C; Snutch and Baillie, 1983). HSF-regulated pathways may well undergo one or more shifts from normal towards more stress-responsive modes across the sub-heat-shock

temperature range (24-28°C), which could in turn lead to confusing results in terms of heat-shock gene expression levels. Notably, this will affect the *hsp16* genes in particular, since these represent the first line of defence against thermal stress in *C. elegans*. Thus *hsp16*::reporter expression cannot provide a reliable biomarker for exogenous stress unless the temperatures of both exposed and control worms are matched very precisely. This requirement was not sufficiently met in our earlier microwave studies. Dawe, p. 14.

However, the small temperature differences described above cannot completely explain the much larger effects previously attributed to microwave exposure (Daniells *et al*, 1998; de Pomerai *et al*, 2000a,b). Because these earlier studies used shielded rather than sham controls, together with an aluminium TEM cell that has not been calibrated by NPL, we suggest that thermal and other extraneous differences may contribute towards the overall effect originally attributed to microwaves. As discussed earlier, only modest adjustment of the normalisation baseline used in Figure 1B is required to generate an apparent 2-fold "microwave induction" after 20 h. We have also previously reported that microwaves can promote protein aggregation in concentrated solutions of bovine serum albumen (de Pomerai *et al*, 2003), when comparing samples exposed in the Cu cell against shielded controls; however, this effect is reduced to insignificance when these microwave exposures are repeated in the Ag cell against sham controls in a Cu cell (Smith *et al*; unpublished data). We no longer have confidence that our original data point to any real non-thermal effect of microwave exposure, and have accordingly submitted a retraction to Nature.

Conclusions:

- Using two matched copper TEM cells, microwave exposure (1.0 GHz, 0.5 W) at 26°C induces expression of a *C. elegans hsp16-1*::reporter construct by ~20% above sham control levels after 2.5 hours, rising to ~50% after 20 hours.
- Calibration of our copper TEM cell has identified an unanticipated power loss problem, which in turn results in slight heating (≤ 0.2°C at 0.5 W) of exposed relative to sham samples.
- Both problems have been ameliorated (by 75% and 50% respectively) following modifications to the TEM cell, including removal of polystyrene packing and silver-plating internal surfaces.
- Sham:sham runs comparing the silver-plated against copper TEM cells show no significant differences in the levels of expression of the stress reporter.
- However, microwave exposure of the same *C. elegans* reporter strain in the silver-plated cell does **not** induce reporter expression above the levels seen in sham controls in the copper cell.
- Slight heating (26.2 versus 26.0°C) can also induce heat-shock reporter expression by ~20%, but a larger temperature rise (to 27.0°C) results in expression levels similar to those at 26.0°C.
- We conclude that *C. elegans hsp16* reporter strains are affected by very small differences in temperature, which can in part explain our earlier findings attributed to non-thermal microwave effects.

Acknowledgements:

The authors are grateful to Mays Swicord and Giorgi Bit-Babik (Motorola Research Laboratories, Florida, USA) for modelling microwave fields within our TEM cell. Although our work was funded by the Mobile Telecommunications Health Research initiative, the views expressed in this paper are the authors' own and do not necessarily reflect those of the funders. We thank Tim Smith and Declan Brady for expert technical assistance, and also four anonymous referees for their critical but helpful suggestions in regard to the content and presentation of this paper.

References:

Adair RK. 2003. Biophysical limits on athermal effects of RF and microwave radiation. Bioelectromagnetics 24: 39-48.

Bohr H, Bohr J. 2000. Microwave-enhanced folding and denaturation of globular proteins. Phys Rev 61E: 4310-4314.

Daniells C, Duce I, Thomas D, Sewell P, Tattersall J, de Pomerai D. 1998. Transgenic nematodes as biomonitors of microwave-induced stress. Mutation Research 399: 55-64.

David HE, Dawe AS, de Pomerai DI, Jones D, Candido EPM, Daniells C. 2003. Construction and evaluation of a transgenic *hsp16*-GFP-*lacZ Caenorhabditis elegans* strain for environmental monitoring. Environ Toxicol Chem 22: 111-118.

de Pomerai D, Daniells C, David H, Allan J, Duce I, Mutwakil M, Thomas D, Sewell P, Tattersall J, Jones D, Candido P. 2000a. Non-thermal heat-shock response to microwaves. Nature 405: 417- 418

de Pomerai D, Daniells C, David H, Allan J, Duce I, Mutwakil M, Thomas D, Sewell P, Tattersall J, Jones D, Candido P. 2000b. Microwave radiation induces a heat-shock response and enhances growth in the nematode *Caenorhabditis elegans*. IEEE Transact Micr Theor Tech 48: 2076-2081.

.de Pomerai D, Smith B, Dawe A, North K, Smith T, Archer D, Duce I, Jones D, Candido EPM. 2003.

Microwave radiation can alter protein conformation without bulk heating. FEBS Lett 543: 93-97.

Hsu A-L, Murphy CT, Kenyon C. 2003. Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science 300: 1142-1145

Leroux M, Melki R, Gordon B, Batelier G, Candido EPM. 1997. Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. J Biol Chem 279: 24646-24656.

Leszczynski D, Joenvaara S, Reivinen J, Kuokka R. 2002. Non-thermal activation of the hsp27/ p38MAPK stress pathway by mobile phone radiation in human endothelial cells: molecular mechanism for cancer- and blood-brain barrier effects. Differentiation 70: 120-129.

Lis J, Wu C. 1993. Protein traffic on the heat-shock promoter: parking, stalling and trucking along. Cell 74: 1-4.

Mutwakil MHAZ, Steele TJG, Lowe KC, de Pomerai DI. 1997. Surfactant stimulation of growth in the nematode *Caenorhabditis elegans*. Enzyme Microb Technol 20: 462-470.

Parsell DA, Lindquist S. 1993. The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual Rev Genetics 27: 437-496.

Power R, David HE, Mutwakil MHAZ, Fletcher K, Daniells C, Nowell MA, Dennis JL, Martinelli A, Wiseman R, Wharf E, de Pomerai DI. 1998. Stress-inducible transgenic nematodes as biomonitors of soil and water pollution. J Biosci 23: 513-526.

Sanders BM. 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit Rev Toxicol 23: 49-75.

Snutch TP, Baillie DL. 1983. Alterations in the pattern of gene expression following heat shock in the nematode *Caenorhabditis elegans*. Canad J Biochem Cell Biol 61: 480-487.

Sulston J, Hodgkin J. 1988. Methods. In "The nematode *Caenorhabditis elegans*" (ed Wood WB), pp 587-606. Cold Spring Harbour Monograph 17, Cold Spring Harbour Press, USA.

Figure Legends:

Figure 1. Microwave effects on *hsp16-1*::reporter expression using matched Cu cells.

PC72 (Part A) or PC161 (Part B) worms were exposed to microwave radiation (1.0 GHz CW, 0.5 W) and sham control (no field) conditions for 2.5 h (Part A) or for up to 20 h (Part B) at 26°C. Reporter activity (β-galactosidase in Part A, GFP in Part B) was determined as described in Methods.

Part A: Both exposed and parallel sham control samples were housed in unmodified Cu cells. Results are shown from 5 independent runs (mean ± SEM from 6 or 24 replicates within each run), all showing modest (~20%) induction of reporter activity in exposed relative to sham samples. In all cases, the left-hand bar in each pair (cross hatched) shows sham levels of reporter activity, while the right-hand bar (speckled) shows the corresponding activity in exposed worms. For all runs, *hsp16-1* reporter activity is slightly but significantly higher in exposed as compared to control worms ($p < 0.01$).

Part B: Time courses for sham and microwave-exposed PC161 worms (as for Part A), kept for 20 h in black non-fluorescent multiwell plates, which were removed at 2- or 4-h intervals (for < 5 min per plate) to allow determination of GFP fluorescence levels, as described in Methods. Solid triangles and solid line, sham; solid squares and dashed line, exposed. Each point shows the mean and SEM from 24 wells. All results were normalised relative to the time zero point for the source population of PC161 worms (100%). Note that sequential points on each curve are **not** independent, but show later fluorescence readings from the same set of wells.

Figure 2. Power loss within TEM cells.

Percentage power loss within the live TEM cell was determined across a frequency range from 50 to 1000 MHz as described in Methods. The upper trace shows the power loss recorded for the original

unmodified copper TEM cell (Cu cell), while the lower trace shows the corresponding power loss in the modified silver-plated TEM cell (Ag cell), following design modifications recommended by NPL.

Figure 3. Temperature calibration of TEM cells.

Temperature increases within both the original Cu (parts A and C) and modified Ag (parts B and D) versions of the TEM cell were measured as described in Methods. Once the initial temperature rise (after switching the power on) had reached a plateau, the rate of cooling was also monitored after switching off the power. Experiments were conducted both at 10 W (partsA, B) and 10.0 W (parts C, D) power input.

Part A (top left): 10.0 W power input, original Cu cell (temperature rise >2.5°C).

Part B (top right): 10.0 W power input, modified Ag cell (temperature rise 1.3°C).

Part C (bottom left): 1.0 W power input, original Cu cell (temperature rise 0.26°C).

Part D (bottom right): 1.0 W power input, modified Ag cell (temperature rise 0.13°C).

Figure 4. Measurement of *hsp16-1*::reporter induction by microwaves in the Ag cell and by heat.

PC72 worms were exposed to microwave radiation (1.0 GHz CW, 0.5 W; Part B only) or to sham control conditions (no field, all parts) for 2.5 h at 26.0°C (all parts), or additionally at 15.0, 26.2 and 27.0°C (Part C). In all cases, reporter β-galactosidase activity was determined as described in Methods.

Part A: Two sets of sham controls were incubated for 2.5 h at 26°C in either an original Cu cell (left-hand bar in each pair – cross hatched) or in the modified Ag cell (right-hand bar – speckled). Results are shown for nine independent runs (mean ± SEM from 24 replicates within each run), and in no case is there a significant difference in reporter activity between the two sham cells ($p > 0.05$).

Part B: Microwave exposures were performed at 26.0°C in the modified Ag cell (right-hand bar in each pair – cross hatched) while shams were housed in an original Cu cell (left-hand bar – speckled). Results are shown for ten independent runs (mean ± SEM from 24 replicates within each run), but in no case is there a significant difference in reporter activity between the sham Cu and exposed Ag cells ($p > 0.05$).

Part C: PC72 worms were placed in incubators held at 15.0, 26.0, 26.2 and 27.0°C (plus controls at 30°C, data not shown) for 2.5 h. Results from 6 independent runs are combined in each bar, normalised

against the overall mean reporter activity at 15.0°C as 100%, and showing SEMs derived from the entire data set (n = 6, since wells within each run are strictly pseudo-replicates). Bars from left to right show reporter expression levels at 15.0, 26.0, 26.2 and 27.0°C. Data from 30°C controls (~1000% relative to 15.0°C) are excluded for clarity. Thermometers and incubators were rotated between runs (see Methods)