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Continuous Wave and simulated GSM exposure at 1.8 W/kg and 1.8 GHz do not induce *hsp16-1* heat-shock gene expression in *Caenorhabditis elegans*

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Running title:- RF does not induce *hsp16-1* gene expression in *C. elegans*.

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Abstract:

Recent data suggest that there might be a subtle thermal explanation for the apparent induction by radiofrequency (RF) radiation of transgene expression from a small-heat-shock-protein (*hsp16-1*) promoter in the nematode, *Caenorhabditis elegans*. The RF fields used in the *C. elegans* study were much weaker (SAR 5-40 mW kg^{-1}) than those routinely tested in many other published studies (SAR ~2 W kg^{-1}). To resolve this disparity, we have exposed the same transgenic *hsp16-1*::*lacZ* strain of *C. elegans* (PC72) to higher intensity RF fields (1.8 GHz; SAR ~1.8 W kg^{-1}). For both continuous wave (CW) and Talk-pulsed RF exposures (2.5 h at 25°C), there was no indication that RF exposure could induce reporter expression above sham control levels. Thus, at much higher induced RF field strength (close to the maximum permitted exposure from a mobile telephone handset), this particular nematode heat-shock gene is not up-regulated. However, under conditions where background reporter expression was moderately elevated in the sham controls (perhaps as a result of some unknown co-stressor), we found some evidence that reporter expression may be reduced by ~15% following exposure to either Talk-pulsed or CW RF fields.

Key Words:- Radiofrequency (RF) fields; heat-shock gene expression; microwave bioeffects; *Caenorhabditis elegans*

Introduction:

Previous reports that weak radiofrequency (RF) fields can induce a non-thermal heat-shock response in the nematode *Caenorhabditis elegans* [de Pomerai *et al*, 2000] have recently been reinterpreted as a subtle thermal artefact caused by very small temperature disparities (≤ 0.2°C) between exposed and sham conditions [Dawe *et al*, 2006]. Using a modified TEM exposure cell, which significantly reduces the temperature differential (to ≤ 0.1°C), this heat-shock response was no longer detectable [Dawe *et al*, 2006]. A similar thermal explanation may also account for the apparent effect of RF fields on mutant phenotype prevalence in temperature-sensitive *C. elegans* mutants grown at intermediate temperatures [Gul-Guven *et al*, 2006]. This is in agreement with biophysical arguments that set limits on possible non-thermal interactions between RF fields and biological systems in equilibrium [Adair, 2003], and with the lack of any generally agreed mechanism for such non-thermal interactions. In the literature, reports of RF effects on heat-shock protein (HSP) expression are divided fairly evenly between those that find no detectable effect [e.g. Cleary *et al*, 1997; Laszlo *et al*, 2005; Lim *et al*, 2005; Lantow *et al*, 2006; Chauhan *et al*, 2006a, b], except at high SARs where thermal effects are clearly implicated [Wang *et al*, 2006], and those where one or more HSPs appear to be induced [e.g. Kwee *et al*, 2001; Shallom *et al*, 2002; Leszczynski *et al*, 2002; Weisbrot *et al*, 2003; reviewed Belyaev, 2005]. Some of these latter reports involve up-regulation of the major inducible HSP70 chaperone, but others focus on the smaller HSP27 protein, whose orthologues are encoded by the *hsp16* gene family in *C. elegans*. RF exposure has also been reported to up-regulate HSP70 protein but not mRNA expression in lens epithelial cells, implicating post-transcriptional controls [Sun *et al*, 2006]. .

Several reports have suggested that some (but not all) vertebrate cell lines may be sensitive to non-thermal effects of RF exposure. These include broad effects on the proteome [Nylund & Leszczynski, 2006] and specifically on the expression of vimentin isoforms [Nylund & Leszczynski, 2004], as well as on the expression and phosphorylation of small heat-shock proteins (HSP27) in human endothelial cells [Leszczynski *et al*, 2002]. However, these changes are not seen in several other cell types [Nylund & Leszczynski, 2006]. RF fields have also been reported to up-regulate expression of HSP70 protein in a p53-deficient mouse embryonic stem cell line [Czyz *et al*, 2004], though this effect was not seen in the presence of functional p53. These findings are supported by several gene-array studies showing significant shifts in the expression of a small subset of genes following RF irradiation [e.g. Remondini *et al*, 2006; Belyaev *et al*, 2006],

although other gene-array studies have reported no significant changes [Qutob *et al*, 2006; Whitehead *et al*, 2006; Gurisik *et al*, 2006]. The RF fields used in these vertebrate cell culture studies were pulsed (GSM) and more intense (SAR ~2.0 W kg^{-1}) than those used in the nematode work (SAR 5-40 mW kg^{-1} CW), raising the possibility that apparent differences between studies on nematodes and on vertebrate cells may reflect modulation and/or dose.

This question could not be addressed using the Nottingham TEM-cell exposure system [Dawe *et al*, 2006], because increasing the input power from 0.5 to 10 W (20-fold) would heat the exposed samples by >1.5°C, even in a TEM cell modified to minimise temperature differentials [Dawe *et al*, 2006]. Therefore, in order to test whether higher radiation doses and/or pulse modulation can cause measurable HSP reporter-gene induction in *C. elegans* in the absence of heating artefacts, we have exposed transgenic PC72 worms (carrying an *hsp16-1*::*lacZ* reporter) to 1.8 GHz fields (both CW and GSM talk-mode) at SAR 1.8 W kg^{-1} for 2.5 h at 25°C in an sXc-1800 exposure system (constructed at IT'IS Foundation, Zurich Switzerland) [Schuderer *et al*, 2002, 2004].

An *hsp16-1* reporter transgene was used here because, like the other *hsp16* small-heat-shock protein genes in *C. elegans*, it is strongly activated by heat at 28°C – which is only 3°C above the upper tolerance limit for this species (standard culture temperatures range between 15 and 25°C). This is in contrast to the major *hsp70* genes, which are only heat-inducible above 33°C [Snutch & Baillie, 1983]. When expressed under stress, the HSP16 proteins form large multimers that act to prevent the aggregation of cellular proteins [Leroux *et al*, 1997] – and indeed HSP16 co-localises with aggregating proteins in worms [Link *et al*, 2006]. It has recently been shown that *hsp16* genes in general (and *hsp16-1* in particular) are key targets for regulation by the DAF-16 FOXO transcription factor in the cell ageing pathway, as well as by HSF-1 in the canonical heat-shock pathway [Hsu *et al*, 2003; Murphy *et al*, 2003]. Promoter deletion studies reveal binding sites for additional transcription factors responsive to ethanol (or general stress) and to hypoxia – albeit by a HIF-1-independent pathway [Hong *et al*, 2004]. These reports strongly imply that *hsp16-1* occupies a central position in a complex network of interlinked stress-response pathways. This underlines the wide applicability of transgenic strains carrying *hsp16*::reporter genes [e.g. PC161, David *et al*, 2003; CL2070, Link *et al*, 1999; PC72, Stringham *et al*, 1994] as general biomarkers of stress, since the nematode HSP16 proteins provide a first line of defence against multiple stresses. These roles may not necessarily be identical for orthologous small hsp's in vertebrates. This study asks whether RF fields at higher SAR can affect *hsp16-1* reporter expression.

<u>Materials and Methods</u>:

C. elegans strain PC72 was generously donated by Professor E.P.M. Candido (Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada) and the *lac*-operon-deleted P90C strain of *E. coli* was originally from Dr A. Chisholm (MRC Laboratory of Molecular Biology, Cambridge, UK). All chemicals were Ultrapure grade from Sigma Ltd (Fancy Road, Poole, Dorset, UK) and all plastic disposables from Nunc Ltd., unless otherwise stated.

Exposure system.

The sXc-1800 exposure system, developed and provided by the IT'IS Foundation and installed at STUK (Helsinki), was employed. This consists of two identical exposure chambers mounted in the same incubator (see Figure 1). It is fully automated and enables well-controlled exposures of cells in monolayers (H-polarization or at H-field maximum of the standing wave) or in suspension (E-polarization or at E-field maximum) at freely programmable amplitude modulations. The exposure chambers are based on resonant R18 waveguides, allowing for SAR values of several hundred W kg^{-1} at the cell with a few watts input power. Identical environmental conditions are achieved in both chambers since the inlet of the airflow through them is at the same location for each. The system monitors the incident field strengths, the proper functioning of the ventilators, the outlet air temperatures and the state of all equipment every 10 sec. The Pt100 temperature sensors (accuracy ± 0.1°C) had been calibrated prior to installation and the recorded differences in temperature are well within the specified long-term stability of the calibration. The induced temperature load due to RF absorption has been characterized as a function of SAR (t) for different signals and volumes of medium. This enables a reliable estimate of the maximum temperature rise as a function of the exposure. Further details of the exposure system can been found in [Schuderer *et al*, 2004]. The ambient ELF exposure was measured in several positions within the incubator using an EFA-3 field measurement system (Wandel & Goltermann, Germany). The signals applied in this study were GSM Talk and continuous wave (CW). GSM Talk is characterized by a random change between the discontinuous transmission mode (DTX) and non-DTX or GSM Basic phases. The distribution in time was exponential with a mean duration of 10.8s for non-DTX and 5.6s for DTX. The dominant modulation components of this signal are 2, 8, 217, 1733 Hz and higher harmonics. More details of the signal are given in [Tillmann *et al*, 2006]. The modulation by data using a random code was not implemented since the associated envelope is composed of higher

frequencies (>100 kHz) that were considered irrelevant for non-thermal effects. For clarity, this simulated GSM exposure is referred to throughout this paper as Talk-pulsed exposure.

Previous dosimetry [Schuderer *et al,* 2004] had to be extended due to the larger dimensions of *C. elegans* compared to cells (the diameter of *C. elegans* L4 larvae is approximately 0.1mm). The dosimetry resulted in an average exposure of the worms of 1.8 W/kg, whereas the gradient in z-direction is about 4 W kg^{-1} mm^{-1}, i.e. the bottoms of the worms were exposed to a SAR of about 2 W kg^{-1} and the tops to 1.6 W kg^{-1}. PC72 displays a moderate roller phenotype due to *rol-6* selection [Stringham *et al*, 1994], and therefore worms would rotate (corkscrew fashion) as they crawl through the K medium across the bottom surface of the dish. It needs to be further noted that the induced E-fields had a pronounced polarization, i.e. parallel to the bottom and in the direction of the waveguide axis. The induced H-fields were approximately 1.6 A m^{-1} and orthogonal to the induced E-field, both of which were dominantly parallel to the bottom of the dish. The temperature load for the experimental conditions was assessed to be approximately 0.04°C for 95% humidity [Schuderer *et al*, 2004], i.e. <0.1°C, at the applied SAR level of 1.8 W/kg. The non-uniformity within the bottom of the dish was determined to be 23% and the relative variability between Petri dishes and experiments was estimated to be less than 5%. The ambient ELF exposure in the incubator in the entire volume of the waveguide was below 5 µT rms; although laboratory ELF magnetic fields might modulate the observed responses to RF, these fields would be experienced equally by both sham and RF-exposed samples, and cannot account for differences between them.

Worm culture and processing.

Cultures of PC72 worms in sealed agar plates were sent by post from Nottingham to Helsinki several days prior to the experiments described here; on arrival, these were placed in a 15°C incubator. Cultures of PC72 worms were synchronised by filtration through a 5 µm mesh filter [Mutwakil *et al*, 1997], and L1 larvae recovered from the filtrate were grown on 9 cm NGM agar plates [Sulston & Hodgkin, 1988] at 15°C until they reached the L4/young adult stage. They were then washed off the plates with ice-cold K medium (53 mM NaCl, 32 mM KCl) [Williams & Dusenbery, 1990] and allowed to settle twice on ice for about 10 min to remove excess bacteria [Dawe e*t al*, 2006]. Equal aliquots of 2-3000 worms were added to 2 ml of fresh K medium in each of the twelve 3.5 cm Petri dishes, which were divided between two 6-dish holders, labelled according to position, and placed inside the upper or lower chambers of the exposure rig. In the randomly selected chamber, the PC72 worms were exposed to an average SAR (at the bottom of

the Petri dish) of 1.8 W kg^{-1} for 2.5 h at 25°C, whilst controls were sham-exposed for the same period at 25°C (no field). The Petri dishes were exposed in H-polarization at a carrier frequency of 1.8 GHz, either non-modulated (CW) or modulated in GSM-talk mode. The experimenter was blind as to which sample-set was exposed and which sham until after the reporter expression analysis had been completed and the codes broken by the IT'IS Foundation. In total, seven CW runs and five GSM talk-mode runs were completed, each involving 6 exposed replicates compared against 6 sham replicates. This work was undertaken at STUK, Helsinki. After exposure, worms were immediately spun down (500 x g for 2 min) and the pellets snap frozen on solid CO_2; all of the frozen worm samples were sent back to Nottingham in an insulated container of solid CO_2 for subsequent reporter analysis, and all remained frozen on arrival. The activity of the PC72 *hsp16-1*::*lacZ* reporter gene (encoding β-galactosidase) was measured on thawed worm samples using the standard MUG assay (with 4-methylumbelliferyl-βD-galactopyranoside as substrate), exactly as described previously [Dawe *et al*, 2006]. Because the P90C food bacteria are deleted for the *lac*-operon, all β-galactosidase activity detected must originate from the *lacZ* transgene carried by the PC72 worms. All sham and exposed samples from each run were processed together (although at that stage, the experimenter did not know which was which), and results are expressed as pmoles of MU reaction product (fluorescent 4-methylumbelliferone) h^{-1} per 1000 worms (mean ± SEM; n = 6). Runs were numbered consecutively, with two runs being conducted each day over six days.

Statistical analysis.

The effects of treatment (RF-exposed versus sham) and radiation type (Talk-pulsed versus CW) on reporter expression (expressed as pmol MU product h^{-1} per worm) were tested using a general linear model in SPSS version 12.0.1 for Windows. A randomized block model was employed because worms in each individual run (block) were subjected to paired exposed and sham treatments. In order to meet the assumption that residuals were normally distributed, raw data were transformed prior to analysis using the following equation:: $x' = (x - 2.5)^3$, where x is a raw datum and x' is a transformed datum. This analysis revealed no effect of radiation type, but inspection of the data revealed that the distribution of background levels of expression was strongly bimodal, with levels of expression being much higher in some runs than others (see Results). Runs showing elevated versus low levels of background expression were therefore analysed separately for an effect of treatment using paired t-tests. In this second analysis, data from the two radiation types were pooled, and no transformation was required.

Results:

RF exposures were performed in a blind manner, using the apparatus illustrated in Figure 1. Computer codes identifying which chamber was sham and which exposed were broken only after completion of all the MUG assays and submission of expression data to the IT'IS Foundation laboratory in Zurich. It later transpired that the upper (slightly warmer) chamber was live (exposed) in all of the Talk-pulsed runs except run 3, whereas the upper chamber was sham in all of the CW runs except run 12. As the recorded temperature difference between upper and lower chambers was independent of exposure/sham configuration (Figures 2B and 3B), it can be concluded that RF did not affect the mean air temperature detectably. The air temperature readings shown would in any case have fluctuated far more than the medium temperature to which the worms were exposed, and the variance seen in Figures 1B and 2B was compounded by the relative inaccuracy of the Pt100 temperature probes used (± 0.1°C). However, the mean difference between the upper and lower chamber temperatures was both consistent (~0.05°C higher in the former) and accurate, being averaged from readings taken every 10 sec during each 2.5 h run. We have previously shown that reporter expression in PC72 worms is up-regulated slightly (~15%; $p < 0.05$) by small increases in temperature of 0.1-0.2°C [Dawe *et al*, 2006], but accurately testing even smaller temperature differences has so far proved impracticable.

Figure 2A shows the reporter expression results obtained from 5 runs using Talk-pulsed exposure. Sham expression was not significantly higher than exposed even in runs 3 and 4, and there was no apparent difference in runs 8-10. We conclude that Talk-pulsed RF exposure at 1.8 GHz does not induce the expression of the sensitive *hsp16-1*::*lacZ* stress reporter, even at a SAR of ~1.8 W kg^{-1}. The miniscule temperature differences (Figure 2B) between upper and lower chambers (~0.05°C) were probably insufficient to affect the expression of this reporter gene, even though it is known to be very sensitive to heat [Dawe *et al*, 2006]. In Figure 3A, the results of 7 CW runs are shown. Once again, reporter gene expression was not significantly higher in shams (runs 1, 7, 11 and 12) or showed no apparent change (runs 2 and 6). In one case (run 5), expression was non-significantly higher in exposed than in sham dishes. Overall, there was no consistent induction of reporter expression by CW RF exposure (1.8 GHz; SAR 1.8 W kg^{-1}), suggesting that the negative conclusions of our previous study [Dawe *et al*, 2006] may also extend to both Talk-pulsed and CW exposures at higher SARs. Using the entire data set for statistical analysis (see Methods), there

was no overall effect attributable to RF exposure ($F_{1, 10}$ = 2.294; p = 0.161), nor was there any interaction between exposure and radiation type (CW versus Talk-pulsed; $F_{1, 10}$ = 0.145; p = 0.712). Moreover, there was no consistent difference between the CW and Talk-pulsed runs ($F_{1, 10}$ = 0.594; p = 0.459). Overall, this pooled analysis suggested no statistically significant effect of RF exposure on *hsp16-1*::reporter gene expression..

However, we noted that the background levels of sham reporter expression within this study fell into two distinct categories: namely, runs with sham expression levels of about 3000 pmol MU h^{-1} per 1000 worms (runs 1-4 and 11-12), and those with much lower expression levels of around 1000 pmol MU h^{-1} per 1000 worms (runs 5-10). This difference accounted for much of the variance in the data set, and could therefore obscure any subtle effects of RF. For comparison, a 2.5 h heat-shock control for this same batch of PC72 worms showed 36-fold induction, with expression levels of 850 (± 3.5 SEM; n = 12) at 15°C versus 31,200 (± 3110 SEM; n = 12) pmol MU h^{-1} per 1000 worms at 30°C (Dawe, unpublished data). Given that reporter expression was only slightly higher at 25-26°C than at 15°C [Dawe *et al*, 2006; see Figure 4C], and that *hsp16* genes remain essentially silent until the temperature exceeds the normal tolerance range for *C. elegans* [Snutch & Baillie, 1983], we suggest that the lower level of background sham expression seen in Figures 2A and 3A probably represents an uninduced state. However, since all reporter expression data have been normalised per 1000 worms, this also implies that the higher level of background sham expression (~3000 pmol MU h^{-1} per 1000 worms) must represent a modest degree of stress-induction – though clearly far less than the high-level induction caused by heat-shock at 30°C over a similar exposure period. The cause of this disparity in background reporter expression levels remains unclear, since it only became evident *post hoc* after completing the experiments. For runs 1-4, conducted early on (during the first 2 experimental days), the L1 larvae used may have hatched from eggs produced under temperature or starvation stress during postage from Nottingham to Helsinki. However, this suggestion cannot explain the equally high background expression levels seen in the final two runs (11 and 12), although it is possible that some other source of stress may have affected all of the worms used in these runs (which were both conducted on the final experimental day). Notably, background expression remained consistently low on days 3 through 5 (runs 5-10). If the overall data set is subdivided into groups on this basis (runs showing modest background expression versus those showing low background expression), there was no significant effect of RF exposure in runs 5-10 showing low background expression (paired t-test; t = 0.868, p = 0.425). However, in runs showing modest background

expression (runs 1-4 and 11-12), RF exposure significantly depressed that expression level by about 15% (paired t-test; $t = -3.953$, $p = 0.011$).

Discussion:

At first sight, this apparent decrease in background expression of the *hsp16-1* reporter appears anomalous, and no strong statistical case can be advanced for this effect within the data set presented here. This is because decreased expression is only detectable after applying an arbitrary *post hoc* grouping of the data on the basis of sham expression levels. Moreover, Talk-pulsed and CW exposures have to be pooled together (as an arbitrary "exposed" category) in order for this effect to show up as statistically significant; sample numbers are simply too small for this difference to emerge if the Talk-pulsed or CW data-sets are analysed separately. If indeed it is true that the lower level of background expression in shams (~1000 pmol MU h^{-1} per 1000 worms) represents an uninduced state of very low expression, then any further repression of reporter activity by RF would be difficult or impossible to detect. This might explain why no such effect was observable in runs where background expression was low. In runs showing moderate background expression, both CW and Talk-pulsed exposures seem to result in quantitatively similar (~15%) decreases in reporter expression, although the data available are too limited to allow proper comparisons between these treatments. Nevertheless, this small decrease does appear to be consistent within this set of experiments, and should therefore not be dismissed out of hand.

One precedent for this in the literature is the decrease in hypoxia protection observed in chick embryos following chronic repeated exposure (30 or 60 min once daily over 4 days) to extremely low-frequency (ELF) or RF fields [Di Carlo *et al*, 2002]. In the case of ELF fields at least, this can be linked to a decrease in HSP70 expression, although this remains to be demonstrated for RF fields. Weak ELF fields (< 5 μT rms) also exist inside the incubator and exposure system, although these should be shielded by the waveguide, and in any case these cannot account for differential effects between RF-exposed and sham samples. However, we cannot exclude the possibility that weak ELF fields might modulate any effect of RF. In the context of the findings reported here, one obvious avenue for future research would be to test PC72 worms that have been deliberately exposed to mild heat stress (e.g. at 27.5 or 28°C) during or prior to microwave exposure at the SAR doses used in this study. However, this might not prove straightforward, given the significant fluctuations in *hsp16-1*::reporter expression seen previously across this temperature range, where expression at 26.2°C was ~15% higher than at either 26.0 or 27.0°C [see Figure 4C in Dawe *et al*,

2006]. Another possibility might be to use a constant temperature (25 or 26°C) together with a defined chemical co-stressor, such as a low dose of cadmium (which is known to induce *hsp16-1* reporter expression strongly) [David *et el*, 2003], either with or without an accompanying RF field. Because this apparent effect of background expression levels emerged only *post hoc* during the data analysis, it was not possible to pursue this question further within the present study. Nevertheless, it remains clear that the 1.8 GHz RF exposures tested here (both Talk-pulsed and CW) are unable to induce *hsp16*::reporter expression in *Caenorhabditis elegans*, even at a SAR dose (~1.8 W kg^{-1}) comparable to that used in other published studies.

Conclusions:

- Using a well-characterised sXc-1800 exposure system (1.8 GHz, SAR ~1.8 W kg^{-1}; 2.5 h at 25°C), expression of a *C. elegans hsp16-1* reporter transgene was not significantly up-regulated by either CW or Talk-pulsed RF fields.
- However, background (sham) levels of reporter expression showed an essentially bimodal distribution, with modest expression in early and late runs but much lower expression throughout the intermediate runs; the reasons for this difference remain obscure.
- In sham runs showing modest expression, reporter activity was consistently repressed by ~15% following RF exposure (whether CW or Talk-pulsed).
- We note that this decrease only became significant after grouping the data arbitrarily on the basis of sham expression levels, and after pooling CW and Talk-pulsed data sets together.
- On this basis, RF exposure at a SAR of ~1.8 W kg^{-1} may possibly cause a modest inhibition of pre-induced heat-shock-reporter expression; future experiments to test this possibility are suggested, but were not possible within the present study because this effect emerged only during *post hoc* data analysis.

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Figure Legends:

Figure 1.

Left: Diagram of the sXc1800 system (E: E-field sensors, T: temperature Pt100 sensors, I_{fan}: fan current sensors, DL: data logger i/o, PC: personal computer via GPIB). **Right**: waveguides mounted inside the incubator.

Figure 2.

Part A shows the activity of an *hsp16-1*::*lacZ* reporter gene measured in PC72 worms after exposure or sham exposure to 1.8 GHz Talk-pulsed fields for 2.5 h at 25°C (simulated GSM; SAR 1.8 W kg^{-1}). Part B shows corresponding mean temperatures (± SD) within the exposed and sham chambers, averaged from chamber temperature readings recorded every 10 sec during the 2.5 h run. Worms were snap frozen after exposure and subjected to MUG assays to determine β-galactosidase activities in each dish, as described in Methods. Each point shows mean and SEM from 6 replicate Petri dishes in each chamber, and data are shown separately for all five runs. In both parts, the shaded left-hand bar in each pair shows data from the exposed chamber (after decoding), and the unshaded right-hand bar in each pair shows sham data from the same run. Run numbers (consecutive, 2 per day) are indicated below the corresponding pair of histogram bars.

Figure 3.

This figure shows similar data from seven CW runs at 1.8 GHz (SAR 1.8 W kg^{-1}) for 2.5 h at 25°C. Mean β-galactosidase activities (from MUG assays; ± SEM) are shown in part A and mean chamber temperatures (± SD) in part B. In all cases, the left-hand shaded bar in each pair shows data from the exposed chamber after decoding, while the right-hand unshaded bar shows the corresponding sham data from the same run. Run numbers are indicated below each pair of bars.