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RESEARCH ARTICLE

Immune responses of a wall lizard to whole-body exposure to radiofrequency electromagnetic radiation

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ABSTRACT

Purpose During the last three decades, the number of devices that emit non-ionizing electromagnetic radiation (EMR) at the wireless communication spectrum has rapidly increased and possible effects on living organisms have become a major concern. The purpose of this study was to investigate the effects of radiofrequency EMR emitted by a widely used wireless communication device, namely the Digital Enhanced Communication Telephony (DECT) base, on the immune responses of the Aegean wall lizard (*Podarcis erhardii*).

Materials and methods Adult male lizards were exposed 24 h/day for 8 weeks to 1880–1900 MHz DECT base radiation at average electric field intensity of 3.2 V/m. Immune reactivity was assessed using the phytohemagglutinin (PHA) skin swelling and mixed lymphocyte reaction (MLR) tests.

Results Our results revealed a noticeable suppression (approximately 45%) of inflammatory responses in EMR-exposed lizards compared to sham-exposed animals. T cell-mediated responses were marginally affected.

Conclusion Daily radiofrequency EMR exposure seems to affect, at least partially, the immunocompetence of the Aegean wall lizard.

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Non-ionizing radiation; inflammation; skin; immunology; reptiles

Introduction

The continuous increase of wireless telecommunication sources, such as mobile phones, cordless phones, wireless fidelity (Wi-Fi) routers, base and frequency modulation (FM) stations, has led to a dramatic increase in environmental levels of radiofrequency electromagnetic radiation (RF-EMR) (Ahlbom and Feychting 2003, Margaritis et al. 2014). These RF-EMR sources emit radiation in a wide spectrum of frequencies (90 MHz – 2500 MHz), with different characteristics regarding their modulation, intensity and energy. The energy of this type of radiation is weak compared to ionizing radiation. Still, it can affect humans and the wildlife (Balmori 2009, Kesari et al. 2013, Singh et al. 2014), even at average electric field intensity values far below International Commission on Non-Ionizing Radiation Protection (ICNIRP 1998) standards, supporting, thus, the notion of RF-EMR non-thermal effects on biological systems (Fragopoulou et al. 2010a, Giuliani and Soffritti 2010).

Recent research provides strong evidence that RF-EMR largely affects critical biological processes and leads to oxidative stress, cell death, nervous system dysfunctions and carcinogenesis (Fragopoulou et al. 2010b, 2012, Sonmez et al. 2010, Kesari et al. 2013, Manta et al. 2014). Such consequences have deleterious effect on wildlife and may contribute to the decline of animal populations (Magras and Xenos 1997, Balmori 2010, Margaritis et al. 2014). However, the potential effects of RF-EMR are still controversial and debated (Miyakoshi 2013). Hence, there is an imperative need for better understanding EMR effects on the processes and mechanisms that are related to the survival of animals, among which the resistance to pathogens and diseases stands out (Ahlbom and Feychting 2003).

To counteract the consequences of pathogens' activity and infectious diseases, vertebrates evolved efficient immune responses, comprising a plethora of non-specific and specific mechanisms (Altizer et al. 2003). New insights into the function of the immune system under non-ionizing EMR exposure demonstrated that electromagnetic fields (EMF) might cause immune dysregulation in humans and the wildlife. Indeed, EMR can alter natural killer cell and macrophage activity and thus, may cause the deviation of inflammatory processes (Rao et al. 1983, Smialowicz et al. 1983, Boscolo et al. 2001, Vianale et al. 2008), trigger oxidative stress affecting susceptibility to diseases (Fernie and Bird 2001, Aydin and Akar 2011), promote cancer development (Repacholi et al. 1997, French et al. 2001), and modify the functional capacity and adhesion ability of

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peripheral blood mononuclear cells (Atasoy et al. 2009). All these important effects have been studied almost exclusively in endotherms, such as birds and mammals (for a review see Balmori 2009).

The ectothermic reptiles differ considerably in terms of physiology and morphology from endotherms (Willmer et al. 2004), mainly in the way they thermoregulate (Avery 1982) and the presence of epidermal scales that cover their body. Nevertheless, the reptilian immune system resembles that of mammals, both structurally and functionally (for a review see Zimmerman et al. 2010). Yet, the impact of abiotic characters that could affect immune function (such as non-ionizing EMR that exists virtually everywhere) remains understudied (Zimmerman et al. 2010). The major ecological significance of reptiles in ecosystems (Kiernan 2014) invites similar studies.

Herein, we investigated the effects of RF-EMR, emitted by a wireless Digital Enhanced Communication Telephony (DECT) phone base, on immune functions of the Aegean wall lizard (*Podarcis erhardii*). We examined both innate (inflammatory) and acquired (T cell-mediated) immune responses to evaluate the effects of EMR on *P. erhardii*'s immunocompetence.

Methodology

Study species and housing

Podarcis erhardii is an insectivorous small-bodied lizard [snout to vent length (SVL) around 70 mm]. Its distribution includes large parts of the Southern Balkans and the majority of the Aegean islands (Valakos et al. 2008). It is a species of particular interest, listed in Annex II of the Bern Convention and Annex IV of the European Union (EU) Habitats Directive.

In this study, we worked with 15 mature male lizards. Animals were captured by noose in the field (Naxos island; 37°02′N, 25°28′E), in accordance with the Hellenic National Legislation (Presidential Decree 67/81), and were transported to the laboratory facilities of the Faculty of Biology at the University of Athens. For each individual we recorded body size (SVL) using a digital caliper (Silverline 380244; accurate to 0.01 mm). Lizards were housed individually in medium sized terraria ($9 \times 15 \times 10$ cm) under a controlled photoperiod (12 h light: 12 h dark) supported by fluorescent tube lighting, whereas additional incandescent lamps (60 Watt) providing heat, allowed animals to thermoregulate for 8 h/day. All lizards had access to water *ad libitum* and were fed once every 2 days with mealworms (*Tenebrio molitor*), coated with a powder supplemented with vitamins and minerals (TerraVit Powder, JBL GmbH & Co. KG).

EMR exposure conditions and dosimetry

We used the base of a typical commercially available DECT phone as radiation emission source. The allocated frequency band for DECT communication is 1880–1900 MHz containing 10 channels of 2 MHz each. During DECT idle operation (i.e. no active telephone call and handset turned off), emission is accomplished randomly in any of the 10 channels in a pulsed manner over the carrier frequency. Pulse duration as recorded by the FSL6 Rohde & Schwartz spectrum analyser (Munich, Germany) was 0.08 msec with a duty cycle of 100 Hz. This means that for every 10 msec, there is an orthogonal burst lasting 0.08 msec. When the base is coupled to a handset (not applied in our set up) (i.e. during a call), there is an additional emission pulse from the base (and the handset) lasting 0.4 msec with the same duty cycle of 100 MHz.

Lizards were randomly divided into three groups of five animals each: (i) Group A comprised sham-exposed animals; (ii) Group B included lizards exposed to DECT base EMR for four consecutive weeks, 24 h/day; and (iii) Group C consisted of lizards exposed to DECT base EMR for 8 consecutive weeks, 24 h/day. Exposed and sham-exposed animals were housed in the same room and under the same conditions (temperature, humidity, sound and illumination), besides radiation exposure. To prevent radiation leakage towards sham-exposed lizards, terraria from exposed and sham-exposed animals were placed separately in specially self-constructed rectangular Faraday cages [85 cm (L) × 32 cm (W) × 32 cm (H)] made of stainless steel screen 16 mesh × 16 mesh. The terraria from exposed animals were circularly moved in respect to the centrally located DECT base, one position every day, so that all animals could be equally exposed to the electromagnetic field.

During the exposure period, electric field intensity was measured using a NARDA SRM 3000 spectrum analyzer (NARDA Safety Test Solutions, Inc., Mönchengladbach, Germany). Measurements of 6-min average [according to ICNIRP's (1998) guidelines] and maximum electric field strength were recorded at different positions of the exposed animals' terraria inside the Faraday cage. Average electric field intensity was 3.2 ± 5% V/m and maximum electric field intensity reached 24.0 V/m.

Specific Absorption Rate (SAR) is the most common dosimetric quantity in the field of a Radiofrequency (RF) source, representing the rate of energy absorption by the tissue and is calculated for a specific point by the equation: $\mathrm{SAR} = \frac{\sigma|\mathrm{E}^2|}{\rho}$, here E stands for the electric field intensity at that point, σ for tissue conductivity (S/m), and ρ for the mass density (kg/m^3) of the tissue. SAR is measured in W/kg. Since, to our knowledge, there are no available data concerning the dielectric properties of lizards' tissues, SAR was not calculated in the present study, contrary to relevant studies that used rodents as an experimental model, where the dielectric properties of their tissues are well characterized (Fragopoulou et al. 2010b, 2012).

Determination of inflammatory and T cell-mediated responses

Immune responses were estimated using the phytohemagglutinin (PHA) skin swelling test (Burnham et al. 2005, Martin et al. 2006) and by one-way mixed lymphocyte reaction (MLR) assays (Lightbody et al. 1971, Smit et al. 1988, Valakos et al. 2007). Though PHA triggers T cell-mediated responses (Goto et al. 1978, Smits et al. 1999), when injected subcutaneously the magnitude of the swelling induced reflects both acquired cell-mediated immunocompetence (Tella et al. 2008) and non-specific macrophage-mediated local inflammation (Martin et al. 2006). On the contrary, in MLR the principal responder population is T cells, which proliferate upon recognition of

xenogeneic major histocompatibility complex (MHC) molecules on stimulator cells and thus, is more specific when testing T cell-mediated responses (Bergholtz et al. 1977).

For PHA challenge, the foot-pad of the right hindlimb of each lizard was injected with 0.05 ml of 2 mg PHA-P (Sigma-Aldrich Chemical Co., St. Louis, MO, L-8754) per ml of phosphate-buffered saline (PBS), pH 7.4 (López and Martín 2005). An equal volume of plain PBS was injected in the left hindlimb as control. The thickness of each foot-pad was measured prior and 24 h after injection, using a micrometer with an accuracy of 0.01 mm. Each measure was taken thrice. The same person (D.M.) performed all injections and swelling measurements to eliminate multiple observers' biases. Reaction to PHA was calculated as the difference of PHA-injected foot-pad thickness 24 h post injection minus the difference of PBS-injected foot-pad thickness 24 h post injection (Belliure et al. 2004, López and Martín 2005). The PHA swelling test was performed on the third, fifth and seventh week of EMR exposure.

In one-way MLR (Lightbody et al. 1971), isolated spleen cells of *P. erhardii* (responders) were co-cultured with inactivated lymphocytes (stimulators) of the donor Skyros wall lizard *Podarcis gaigeae*. These two species exhibit a genetically diverse MHC phenotype (K. Sagonas and A. Runemark, unpublished data). The experiment was carried out during the 4th and 8th week of continuous exposure. Both MLR experiments were conducted a week after the PHA swelling test, to ensure that T cell responses would not be affected by PHA-induced inflammation. Spleen cell isolation was performed as described (Valakos et al. 2007) and cells were adjusted to 2×10^6 splenocytes/mL complete medium (CM), consisting of Roswell Park Memorial Institute (RPMI) – 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1% fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Sigma-Aldrich) 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 5×10^{-5} M 2-mercaptoethanol and 1% penicillin-streptomycin (all from Gibco). For inactivation, *P. gaigeae* splenocytes were suspended in RPMI-1640 and treated with 25 μg/ml mitomycin-C (mit-C; Kyowa, Japan) which prevents DNA replication and, hence, proliferation (Bach and Voynow 1966) at 37 °C for 45 min. Mit-C was inactivated by adding FCS and splenocytes were washed thrice in CM by centrifugation at 300 *g* for 5 min at 25 °C. Two $\times 10^5$ responder splenocytes from each animal in 100 μl aliquots were mixed with an equal number of stimulator splenocytes at a final volume of 200 μl/well. Unmixed (2×10^5 cells/well) responder and stimulator splenocytes from *P. erhardii* and *P. gaigeae*, respectively, were used as controls. Assays were performed in quadruplicates in 96-well U-bottom tissue culture plates (Costar, Cambridge, MA, USA). Cultures were incubated for 5 days at 37 °C, 5% CO_2. During the last 18 h, 1 μCi [^{3}H]-thymidine (The Radiochemical Center, Amersham, Bucks, UK) was added to each culture and cells were harvested using a semi-automated cell harvester (Skatron Inc., Tranby, Norway). The radioactivity incorporated into cellular DNA was determined by liquid scintillation counting. Data were expressed as counts per minute (cpm) and stimulation indices (SI) were calculated according to the following equation:

$$\text{S.I.} = \frac{\text{cpm MLR culture}}{\text{cpm of responder cells} + \text{cpm of stimulator cells}}$$

Infestation levels

To control for the effect of parasites on lizards' immune responses, the percentage of infected cells on a total of 10,000 red blood cells was estimated (hereinafter referred as infestation levels). A blood sample from each lizard was obtained immediately after capture by clipping off the tip of the tail. Blood smears were prepared on microscope slides, air dried, fixed in absolute methanol for 10 min and stained for 20 min with Giemsa (Sigma-Aldrich) diluted 1:9 (volume/volume) in PBS, before examination for the presence of intra-erythrocyte parasites. Samples were analyzed using an optical microscope at 100×. The haemoparasites identified were haemogregarines.

Statistical analyses

Prior to analyses, we tested the normality and heteroscedasticity of the data. When parametric assumptions were not met, non-parametric tests were performed. To examine the effects of PBS- versus PHA-injection on foot-pad thickness (swelling test), we used the Wilcoxon matched paired test. We performed the Mann-Whitney U test to investigate the effects of EMR on lizard's immune responses, using limb swelling 24 h post injection and/or SI values as dependent and EMR treatment (exposed versus non-exposed) as independent variables. Moreover, given the effect of parasite burden to cell-mediated responses (Råberg et al. 2009), we applied non-parametric analysis of covariance (ANCOVA) (i.e. rank ANCOVA; RANCOVA) (Quade 1967), using infestation levels between EMR-exposed and sham-exposed lizards as covariate. We also used RANCOVA to test possible effects of SVL on cell-mediated responses (Sacchi et al. 2007), using the thickness of the limb or SI values as dependent variables and treatment as independent variable. Finally, we conducted a Kruskal-Wallis analysis of variance (ANOVA) to examine the effects of long-term EMR exposure on cell-mediated responses. For this analysis, PHA-limb-swelling or SI values were used as dependent variables, whereas the time-points tested (i.e. day/week of experiment) as independent variable. All statistical analyses were carried out using SPSS v.22.0 (IBM SPSS Statistics; Corp Released 2013).

Results

Effect of DECT base radiation on swelling response

PHA injections induced a significant swelling in lizards' limbs as indicated by the comparison of foot-pad thickness prior to and after injection ($Z = 3.41$, $p < 0.001$; Table 1). On the contrary, we did not observe significant inflammatory responses caused by PBS injections ($Z = 1.48$, $p = 0.139$) in all lizards examined.

The comparison of PHA-swelling responses between EMR-exposed and sham-exposed lizards prior to EMR exposure (day 0 hereinafter) showed no significant differences ($Z = 1.29$, $p = 0.198$), indicating that at the beginning of the experiment (baseline) the three lizard groups had similar immune

Table 1. Results of the descriptive statistics for PHA swelling response and stimulation indices (SI) values for sham-exposed and EMR-exposed lizards.

Trait	sham-exposed	EMR-exposed
Swelling day 0 (PHA)	0.44 ± 0.05; (*5*)*	0.35 ± 0.02; (*10*)
Swelling week 3 (PHA)	0.49 ± 0.04; (*5*)	0.22 ± 0.02; (*10*)
Swelling week 5 (PHA)	0.44 ± 0.06; (*3*)	0.18 ± 0.02; (*5*)
Swelling week 7 (PHA)	0.44 ± 0.07; (*3*)	0.17 ± 0.02; (*5*)
SI week 4 (MLR)	1.82 ± 0.24; (*2*)	1.58 ± 0.17; (*5*)
SI week 8 (MLR)	2.07 ± 0.19; (*3*)	1.59 ± 0.18; (*5*)
SVL	71.90 ± 0.75; (*14*)	72.44 ± 1.13; (*10*)
Infestation levels	0.0002 ± 0.00004; (*5*)	0.0002 ± 0.00008; (*10*)

PHA, phytohemagglutinin; SI, stimulation index; MLR, mixed lymphocyte reaction; SVL, snout to vent length;
*mean ± standard error (*number of animals*).

responses. However, exposure to EMR caused a significant reduction of inflammatory responses, with EMR-exposed lizards demonstrating a tremendous suppression of their inflammatory response (∼45%) compared to sham-exposed animals (3rd week: $Z = 3.06$, $p = 0.002$; 5th week: $Z = 2.24$, $p = 0.025$; 7th week: $Z = 2.24$, $P = 0.025$; Figure 1). These differences persisted in repeated analysis considering SVL (3rd week: $F_{1,12} = 26.53$, $p < 0.001$; 5th week: $F_{1,5} = 19.74$, $p = 0.007$; 7th week $F_{1,5} = 16.71$, $p = 0.009$). When infestation level was taken into account, the differences remained (3rd week: $F_{1,12} = 31.33$, $p < 0.001$; 5h week: $F_{1,5} = 15.74$, $p = 0.011$; 7th week $F_{1,5} = 13.05$, $p = 0.015$) and the presence of haemogregarines seemed to have no effect on *P. erhardii*'s EMR-induced immune suppression.

The comparison of PHA-swelling response on day 0 and after 3, 5 and 7 weeks of continuous EMR exposure yielded significant differences (Group B: $Z = 1.98$, $p = 0.048$ and Group C: $\chi^2 = 10.68$, $df = 3$, $p = 0.014$), with EMR-exposed lizards denoting significant decline in their immune response (Figure 1). Though the suppression of inflammation at the 7th week of the experiment (∼51%) was greater than at the fifth (47%) and third (40%) weeks, this decline was not statistically significant ($\chi^2 = 2.80$, $df = 2$, $p = 0.247$). Finally, we observed no significant differences in foot-pad thickness during the course of the experiment in sham-exposed lizards ($\chi^2 = 2.20$, $df = 3$, $p = 0.532$; Figure 1).

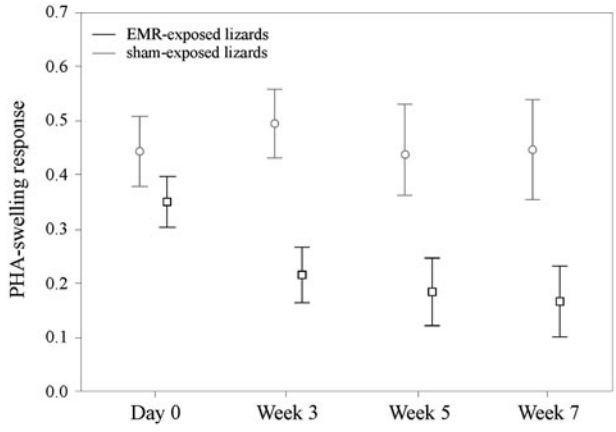

Figure 1. Reaction to PHA between EMR-exposed and sham-exposed lizards during the 3rd, 5th and 7th week of the experiment. The swelling value was calculated as the difference of the thickness of the PHA-injected foot pad 24 h after injection minus the difference of the thickness of the PBS-injected foot pad 24 h after injection. Points represent means and vertical bars standard error.

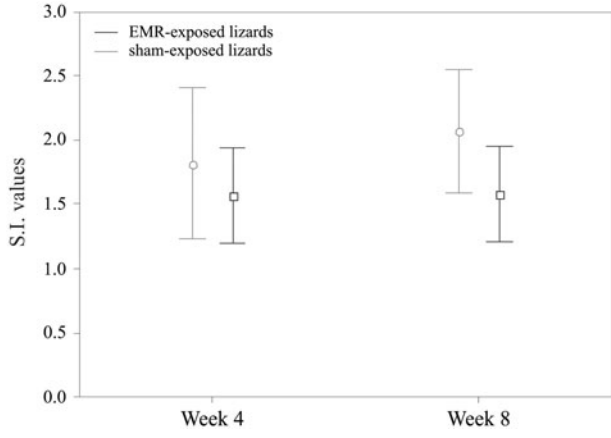

Figure 2. Stimulation indices of EMR-exposed and sham-exposed lizards as estimated by one-way MLR during the fourth and eighth week of the experiment. Points represent means and vertical bars standard error.

Effects of DECT base radiation on T cell-mediated response

The analysis for SI of one-way MLR assays between exposed and sham-exposed lizards did not reveal any differences (4th week or Group B: $Z = 0.39$, $p = 0.699$ and 8th week or Group C: $Z = 1.64$, $p = 0.101$), with EMR-exposed and sham-exposed lizards showing similar T cell-mediated responses (Table 1 and Figure 2). Moreover, we did not observe any differences in the lymphoproliferative response between the two time-points tested (weeks 4 and 8; $Z = 0.104$, $p = 0.917$), or between haemogregarin infected and non-infected animals (data not shown).

Discussion

It has long been argued that the metabolic activities of the immune system, and in particular that of lymphocytes, are usually the target of non-ionizing EMR (Czerski 1975, Lushnikov et al. 2003, Balmori 2009). Additional research, however, showed that EMR does not affect, in a similar way, the ability of all leukocyte populations to respond to subsequent challenges/stimulations (Wiktor-Jedrzejczak et al. 1977, Conti et al. 1983, Di Giampaolo et al. 2006). As in other biological systems (e.g. nervous, reproductive), these observations are further complicated given the non-linear correlation of EMR strength with end-results, and the variable contribution of several additional factors (e.g. model systems used, exposure set-up and/or characteristics of the EMR source) to the final outcome (Margaritis et al. 2014). To date, the knowledge of RF-EMR impact on the immune system is limited and data are derived from in vitro cultures of human cells and in vivo studies in rodents (Gatta et al. 2003, Sambucci et al. 2011, Bhattacharya and Roy 2013).

Lizards represent a good model system for inflammation studies (Alibardi 2010), since, though they are ectotherms, their immune system comprises both innate and adaptive defence mechanisms. Here, we studied the effect of RF-EMR on lizard

immunity; we assessed possible alterations in their immunocompetence due to exposure to 1880–1900 MHz DECT base RF-EMR. Our findings suggest that this type of radiation specifically suppresses acute inflammation in lizards, but does not affect the T cell compartment. Specifically, EMR-exposed *P. erhardii* lizards exhibited 45% reduction in their non-specific inflammatory responses compared to sham-exposed lizards, but a similar effect was not unveiled when immunoreactivity and proliferative responses of T lymphocytes were analyzed (SI values; Figure 2).

During PHA-induced skin swelling, two distinct phases of immune response occur: The first involves exudation of plasma and local accumulation of innate leukocytes, and the second, that occurs approximately 20 h after injection, comprises site infiltration by T lymphocytes (Stadecker et al. 1977, Goto et al. 1978, Martin et al. 2006). Since EMR-exposed lizard T lymphocyte proliferation was not immunocompromised, a scenario explaining the suppression of acute inflammation would seemingly involve alterations in the functional activity of phagocytic cells and the cytotoxic potential of neutrophils under the influence of EMR (Lushnikov et al. 2001,2004). Nevertheless, such alterations are unlikely to be explained on the basis of thermal mechanisms, since the RF-EMR used herein does not increase the body temperature of exposed animals (non-thermal effects; Fragopoulou et al. 2010a, Giuliani and Soffritti 2010). Apparently, RF-EMR rather inhibits processes involved in inflammatory responses, such as the synthesis of metabolites like cyclooxygenase-2 and/or arachidonic acid-derived products, leading to suppression of the phagocytic activity (especially of neutrophils) and/or reduced phagocyte infiltration in the inflamed area (Popov et al. 2001, Kolomytseva et al. 2002, Lushnikov et al. 2004, Gapeyev et al. 2006). Accordingly, the dynamics of the first acute inflammation phase following PHA injection was altered and edema suppression was observed.

On the contrary, our results failed to support any effects of RF-EMR on T lymphocyte activity. This finding is in agreement with other studies in rodents (Wiktor-Jedrzejczak et al. 1977, Nageswari et al. 1991). Two possible explanations are suggested. The first relates to differences in RF-EMR penetration through the lizard's body. We believe that the presence of scales and the thick keratinized epidermis of lizards do not allow radiation to affect internal organs that lie deeper in the body cavity (Gabriel et al. 1996, Vitt and Caldwell 2014). As such, energy absorption is superficial and the skin absorbs more than the spleen. Though this scenario is plausible, it is hardly supported due to the lack of data on lizard tissue dielectric properties. The second explanation is related to the small size of T lymphocytes and their continuous mobility through the circulation to secondary lymphoid organs including the spleen (Zimmerman et al. 2010). These features lead to reduced exposure of T cells to RF-EMR compared to tissue-resident macrophages that are bigger in size and rather stationary in the skin. It is possible that any animal species' immunology may be affected by long or frequent EMF exposures of real pulsed communication signals, so further studies that will include additional species and more detailed immune analyses are required to elucidate the mechanisms underlying our observations.

In conclusion, under the specific exposure conditions, RF-EMF DECT base radiation has a moderately harmful effect on the immune system of *P. erhardii*, affecting in principle the non-specific inflammatory response. Since a number of infectious diseases and parasites initially elicit inflammatory reactions, these pronounced deficient inflammatory effects of RF-EMR might increase the susceptibility of lizards to infections and, hence, jeopardize population's conservation status.

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Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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