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Immune stimulation in fish and chicken through weak low frequency electromagnetic fields

J. J. M. Cuppen · G. F. Wiegertjes · H. W. J. Lobee · H. F. J. Savelkoul · M. A. Elmusharaf · A. C. Beynen · H. N. A. Grooten · W. Smink

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Abstract A hypothesis is proposed how Low Frequency Electromagnetic Field (LF EMF) exposure can stimulate an immune response, based on recent insights in immunology. We hypothesize that the Immunent EMF treatment induces mild stress to cells, which then produce cytokines that function as alarms or so called danger signals for the immune system. In this way EMF treatment takes the place of multiplying pathogens, and the damage these cause, in the triggering of an immune response. In a first series of experiments in vitro common carp head kidney-derived phagocytes were used to determine ROS production as a measure for immune activation. Exposure to LF EMF signals (200–5,000 Hz) at 5 μT or 1.5 mT led to 42 or 33% increase in immune activity, respectively, compared to negative control values. EMF could also additionally stimulate chemically pre-stimulated samples up to 18% (5 μT) or 22% (1.5 mT). Significance of increase in ROS production in the total series was: $p < 0.0001$. In a second series of experiments in vitro commercial goldfish were used. Groups of fish were housed under equal conditions in at least four control tanks and 8–16 EMF-exposed tanks. Exposure was done with a predominantly vertical field at field strengths (rms) between 0.15 and 50 μT. Without treatment mortality was about 50% after 18 days, while the treatment at 5 μT reduced it to 20% on average. At field strengths 0.15, 0.5, 1.5, 5, 15 and 50 μT an equally strong effect was found. Reducing the field strength to 0.05, 0.06, 0.01 and 0.003 μT showed a gradually decreasing effect, which only at 0.003 μT is no longer statistically significant. Finally, in vitro experiments were done with 560 commercial broiler chickens exposed to infection pressure from coccidiosis. EMF exposure at 6.5 μT reduced intestinal lesions by 40% and improved feed conversion by 8%.

Keywords Immune stimulation · EMF · ELF · Electromagnetic fields · Feed conversion

J. J. M. Cuppen (✉) · H. W. J. Lobee
Immunent BV, Veldhoven, The Netherlands
e-mail: jcuppen@immunent.com

G. F. Wiegertjes · H. F. J. Savelkoul
Wageningen University, Wageningen, The Netherlands

M. A. Elmusharaf · A. C. Beynen
Utrecht University, Utrecht, The Netherlands

H. N. A. Grooten · W. Smink
FIS BV, Utrecht, The Netherlands

1 Introduction

Simkó et al. (2001) and Lupke et al. (2004), have demonstrated that human and murine macrophages can be stimulated to higher activity through LF EMF.

Several authors (see Blank et al. 1992; Goodman et al. 1994; Blank et al. 1995; Simkó and Mattsson 2004; Monselise et al. 2003; De Bruyn and de Jager 1994; Mevissen et al. 1998; Markov et al. 2006; Cossarizza et al. 1993; Cuppen and Vink 2004) discussing the effects initiated by various EMF signals have demonstrated the production of cytokines, increased immune parameters and stress effects and concluded that EMF causes stress at the cellular level and that this leads to production of cytokines and consequently biological response, including immune response. This has led Simkó and Mattson (2004) to formulate a hypothesis that proposes a mechanism for immune activation due to short term EMF exposure (see Fig. 1) and a mechanism for cell damage due to long term EMF exposure.

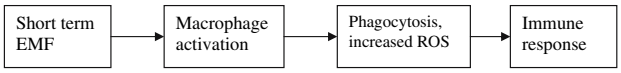

Fig. 1 Simkó and Mattsson hypothesis

During the last decade in immunology a hypothesis has been developed that immune response is not only due to the presence of non-self cells but also needs the presence of promoters (e.g. Matzinger 2002; Kapsenberg 2003; Shi et al. 2003). This holds in particular for the innate immune response to pathogens not earlier encountered. Such promoters are called ''danger signals'' in immunology. Examples might be Heat Shock Proteins (prime examples of cell response to injury or insult), as well as Interferon, Interleukins and other cytokines. Danger signals are formed when cells get stressed or damaged, and this is ''presumed by the cells'' to be caused by harmful pathogens. The idea behind the view is that strange DNA that does not cause damage, such as a foetus or food, does not and should not trigger an immune response.

In immunology other factors besides damage due to pathogens have been identified that stimulate the production of danger signals, such as stress or shock from salt, cold or heat. See Engelsma et al. (2003), and Huising et al. (2003).

The danger signal hypothesis can be schematically represented as in Fig. 2.

The research reported here explores effects on the immune response to various pathogens of exposure to a composite LF EMF signal containing frequencies between 250 and 5,000 Hz, with a strength from 3 nT to 50 μT given for 30 min per day. In experiments, in vitro, with immune cells from carp, and in vitro both with goldfish and chicken broilers significant and often strong effects of, probably, immune stimulation have been observed. The effects include: 40% increased ROS production by isolated phagocyting cells ($p < 0.001$), 60% decreased mortality in infected fish ($p < 0.001$), 8% decreased feed consumption for equal growth in chicken ($p < 0.05$) comparable to the use of preventive antibiotics and 40% decreased intestinal lesions in chicken ($p < 0.01$) due to Coccidiosis infections.

We propose a hypothesis for a working mechanism of EMF on the immune system of this broad range of animals. The hypothesis regards the possible mechanism of action and attempts to answer the question how such weak, short duration EMF signals can induce an effective immune response.

Subsequently we discuss the experimental results in cells and goldfish in detail.

2 Hypothesis

Based on published theoretical and experimental studies, as well as our own experience we propose a hypothesis, that combines and extends the views quoted above, by

(a) the observation that an immune response is a self stimulating process, at least in the initial stages. Macrophages become activated under the influence of danger signals, and thus have a higher statistical chance and a lower expected time until they actually encounter and recognize pathogens. Once this happens they themselves produce cytokines that increase the immune response etcetera,

b) the observation that for chronic infection apparently the feedback cycle stated in (a) does not gain sufficient momentum for the infection to reach acute phase,

c) the observation that for new infections a more active and alert immune system will start the feedback loop in (a) earlier, thus slowing down the multiplication of pathogens and reducing damage.

Therefore we propose an extended hypothesis which can be schematically represented as in Fig. 3.

The core of the proposed Hypothesis is that short term LF EMF exposure, possibly repeated, can produce danger signals that can trigger the immune system activation feedback mechanism in the presence of pathogens. As such the EMF induced danger signals take the place of the danger signals that would be produced by multiplying pathogens and the damage they cause to cells and tissues. In this way a timely EMF treatment can avoid the delay given by the time it takes for enough damage to occur and danger signals to be produced in a normal disease development. Thus the immune response to pathogens can be

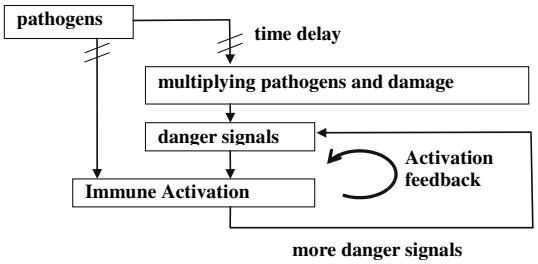

Fig. 2 Matzinger hypothesis

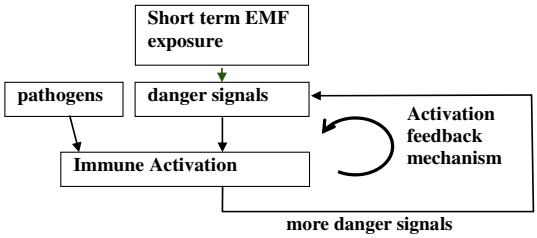

Fig. 3 Our hypothesis

advanced in time and damage and a well developed pathogen attack can be avoided.

In our own in vitro experiments (reported below) we have observed immune stimulation effects after repeated exposures of 30 min per day. There is evidence in literature and there are anecdotal indications that continuous exposure to EMF leads first to increased immune parameters, but later to decreased immune parameters, which can be interpreted as exhaustion (Mevissen et al. 1998; Jager et al. 2004).

3 Materials and methods

3.1 In vitro experiments

Common carp head kidney-derived phagocytes were used. The objective of this test is to measure the modulation of the ROS production in macrophages and neutrophilic granulocytes of the carp pronephros, or head-kidney.

The measurement is based on the reduction of a salt: Nitro Blue Tetrazolium (NBT) by O_2^-. This results in a blue coloration, which is measured spectrophotometrically.

3.1.1 Biochemical materials

Phorbol 12-myristate 13-acetate (PMA), 0.01 μg/μl, a standard chemical stimulant of ROS production in leukocytes known to give a high response as a positive control; RPMI without phenol red: washing and culture media; Nitro Blue Tetrazolium in RPMI without phenol red. (NBT; grade III) 1 mg/ml final concentration; 2N KOH (Potassiumhydroxid); 100% and 70% methanol; Dimethylsulfoxide (DMSO); flat bottomed 96-well microtiterplate (sterile) coated with adherent cells; spectrophotometer with filter at 690 nm.

3.1.2 Biochemical methods

Leukocytes are collected from the head kidney and stored at 4°C overnight. Next, they are plated out in 96 well trays with 100 μl in each well. The cells are allowed to adhere for 30 min at 27°C (5% CO_2, humidity 100%). After inspection, non-adherent cells are washed away by rinsing twice with 100 μl of RPMI-phenol red culture medium (one row at a time).

The NBT assay is started by removing the supernatant and by adding 150 μl of NBT and subsequently the stimulators (10 μl of PMA and/or 30 min EMF), followed by or, in case of EMF, in parallel to incubation of the cells for 90–120 min at 27°C (5% CO_2, humidity 100%).

After the incubation, the NBT solutions are removed and the cells are washed once by rinsing with RPMI medium without phenol red. The remaining cells are fixated for 5 min with 100 μl 100% methanol, then washed twice with 100 μl 70% methanol to remove possible NBT-rests. The plate is left to dry.

The blue formazan inside the cells is solubilized in 100 μl 2N KOH to which 100 μl DMSO is added. The content of the well is mixed thoroughly.

The optical density is measured at 690 nm (ref. filter at 414 nm) in a spectrophotometer.

3.1.3 EMF materials

An exposure magnet was designed for exposing one or two rows of a 96 well plate simultaneously. Since earlier experience indicated cross talk to controls at enormously low field strengths we aimed for the lowest fringe fields possible. Based on experience from MRI magnets, Ferrite C arm units were built for exposing one or two rows of a 96 well plate to a uniform AC magnetic field. The ''C'' of the magnet was built of 16 mm^2 Ferrite 3C90, the pole shoes were $99 \times 30 \times 8$ mm. Above and below the pole shoes, coils with 600 turns each were placed around the C arm. Saturation in the ferrite occurs above 170 mT, therefore this setup allowed a field strength of 5 mT to be achieved in the exposure gap without saturation effects in the C. A uniformity of 10% or better was achieved at the well positions.

Side ways, the fringe field was measured to decrease by at least a factor 2 every 2 cm. Thus at a distance of 5 m (which was kept for the controls) one may safely assume the fringe field to be below 1 pT. It was no longer measurable with our equipment at 1 m distance.

Signals were generated by an Immunent signal generator, capable of generating field strengths between 0.15 μT and 1.5 mT in the exposure area, with a composite LF EMF signal containing shaped waveforms with base frequencies between 250 and 5,000 Hz. This generator is available from Immunent BV.

The signal generator monitored currents through the coils during the experiment, which were calibrated to correspond to the desired field strength using an F.W.Bell 7000 Tesla Meter with sensitive probe.

3.1.4 Experiment design

Each run measured 48 samples, in six groups of 8 samples. Half of the groups were not chemically pre-stimulated (negative) and half were pre-stimulated with PMA (positive). Two groups were taken as controls, while EMF stimulation was done on four groups, two at 5 μT field strength and two at 1.5 mT field strength.

Exactly those runs were included in the results where the positive control average exceeded the negative control average by at least 50%, independent of the measurements

for the EMF exposed cells. If that is not the case it must be assumed that the cell isolation was not properly done. This led to the exclusion of 6 runs out of a total of 20 runs executed.

3.2 In vitro experiments

Subsequently, a series of experiments were performed using fantail goldfish (*Carrassius auratus* spp.), heavily infected with ecto parasites (gill parasites) such as *dactylogyrus/gyrodactylus, trichodina, chilodinella* and *costia*. Infection with those parasites occurs consistently at the grower and increases during storage, packing and international transport due to crowding. This and subsequent secondary bacterial infections cause high mortality if not treated. The progress of the diseases can be measured by daily counting and removing dead fish over a period of a few weeks. Fish were randomly allotted over the tanks. Individual fish weight was 2.5 g (±0.5 g). Daily, the fish were checked for health. Dead fish were counted, removed and discarded.

The number of fish per replicate tank varied between the experiments, 12 in the first and second experiment, 21 in the third, and 30 in the fourth.

Fish were housed in PVC tanks with a content of 30 l. Aeration of water and daily change of 20% of the water maintained water quality. Fish were fed commercial feed ad libitum.

Groups of fish were housed under equal conditions in at least 4 control tanks and 8–16 EMF-exposed tanks. Exposure was done at field strengths between 0.15 and 50 μT by coils positioned outside of the fish tanks, creating a predominantly vertical field. Lower field strength exposures were achieved by placing additional tanks in the stray field of a tank with a coil, calibrating that stray field to the current in the coil, and then controlling the current.

The tanks were standard PVC tubs of 50 cm height, 30 cm diameter, around which standard isolated 1.5 mm^2 electricity wire was tightly wound in 160 turns. At the lower end a spiral end cap of 12 turns over the outer half of the diameter and at the top flange 20 additional turns were wound to optimize uniformity in the tub. Thus inside the tubs a practical, but close approximation was achieved of the field of a long solenoid coil. The deviation of the theoretically uniform field of the solenoid was calculated using Vector Fields, and is better than 5% in the water volume further than 4 cm from the lower outside circumference of the tub, and better than 20% further than 2 cm from that outside circle.

The signal generator was as described above for the in vitro experiments.

Finally, experiments were done with commercial chicken broilers, exposed to Coccidiosis, which is a common infectious disease in poultry, causing major economic losses. For two subsequent experiments 288 and 272 one-day old female broilers (Ross 308) were purchased from the hatchery and randomly housed in wire-floor, suspended cages. Each test cage was provided with a 16 loop coil under the floor through which LF EMF treatment was administered for 30 min per day. The field strength (rms) in the middle of the cage was set at 6.5 micro Tesla. Intestinal lesion scorings were performed blindly to the treatment modality on days 15, 29, and 36 (days 6, 14, and 21 PI). Two birds per cage (32 cages) were euthanized by cervical dislocation, dissected and different coccidial lesions were scored. Feed conversion is calculated as the feed intake (kg) divided by the growth (kg) over the total period. Details of the experimental setup are available in Cuppen et al. (2006).

4 Results and discussion

4.1 In vitro experiments

ROS measurements were averaged over the eight samples available in each group. Averages of the measurements without pre-stimulation and of the positive control group (with pre-stimulation) were divided by the negative control average in the corresponding run. The averages of pre stimulated measurements were divided by the corresponding positive control average. The resulting data, with standard deviation estimates derived from the data in each group, are given in Table 1.

ROS production was increased in 12 out of 14 cases after 5 μT treatment, in 11 out of 14 cases after 1.5 mT treatment. With pre stimulation, ROS production was further increased in 9 out of 14 cases at 5 μT, and in 12 out of 14 cases at 1.5 mT.

On average, exposure to 5 μT or 1.5 mT led to 42 or 33% increase in immune activity, respectively, compared to negative control values. EMF could also additionally stimulate PMA-stimulated samples up to 18% (5 μT) or 22% (1.5 mT). Statistical analysis was carried out with SAS via a meta-analysis of the 14 datasets. A significant ($p < 0.0001$) increase on the response of the EMF exposed cells was found.

These data are in line with those reported for 50 Hz, sinusoidal, 1 mT fields in murine and human macrophages in Simkó et al. (2001) and Lupke et al. (2004).

4.2 In vivo experiments goldfish

The subsequent series of experiments with diseased goldfish returned the following results. In each graph mortality is indicated in % of the total number of fish in each test group, as it develops over the course of the experiment. As

Table 1 ROS production results of 14 runs with six times eight samples each

	% average normalized to negative control average						% average normalized to positive control average					
	Neg contr		Neg, 5 μT		Neg, 1.5 mT		Pos contr		Pos, 5 μT		Pos, 1.5 mT	
	Av (%)	SD (%)	Av (%)	SD (%)	Av (%)	SD (%)	Av (%)	SD (%)	Av (%)	SD (%)	Av (%)	SD (%)
v50131	100	8	109	6	95	7	223	41	99	13	87	10
v50202	100	6	131	23	108	8	162	30	117	19	103	30
v50209	100	24	138	36	142	23	418	73	115	9	190	17
v50215	100	54	278	120	176	131	788	118	98	17	113	46
v50222	100	24	118	41	115	50	319	59	130	25	144	32
v50223	100	50	75	17	151	32	235	90	136	22	104	24
v50315	100	10	120	25	62	18	166	28	131	34	106	21
v50405	100	15	128	29	112	31	162	23	141	42	141	31
v50406a	100	17	142	25	152	23	179	37	143	26	126	16
v50406b	100	31	206	119	322	70	230	33	143	54	175	26
v50419a	100	28	93	27	66	11	201	48	92	20	101	14
v50419b	100	10	212	50	138	40	259	54	133	26	110	17
v50420	100	15	112	27	118	21	205	38	86	17	122	14
v50426	100	55	126	89	112	67	323	86	82	13	92	19
Average	100		142		133		276		118		122	

can be seen the horizontal mortality lines after day 20 in experiment 3 (no further mortality), after 18–20 days usually the surviving fish had recovered from the diseases both in the control and the test groups, as was confirmed by visual inspection. Most test groups consisted of at least four replicates, in which the number of fish initially was gradually increased after more experience was gained. For some experiments however with gradually varying parameters two replicates were taken.

Please note that in each experiment a new batch of fish was procured from the importer of the fish. Fish from groups heavily infected with parasites were selected. However the initial disease/health level of the fish varies between experiments. The absolute levels of survival between experiments are therefore not comparable. Within one experiment the fish had comparable health so the comparisons within one experiments are valid.

The first experiment compared survival in four control groups with eight groups treated at 5 μT. Each group contained 12 fish (Fig. 4).

As can be seen there is a large difference in the survival, which was consistently more than 80% better in the treated group over the course of the experiment. The end values were 52 ± 14% untreated and 19 ± 10% treated. The experiment was repeated, this time with 12 treatment groups of 12 fish each.

Data in Fig. 5 shows that the improvement was again a factor 2. The end values were 52 ± 8% untreated and 26 ± 19% treated.

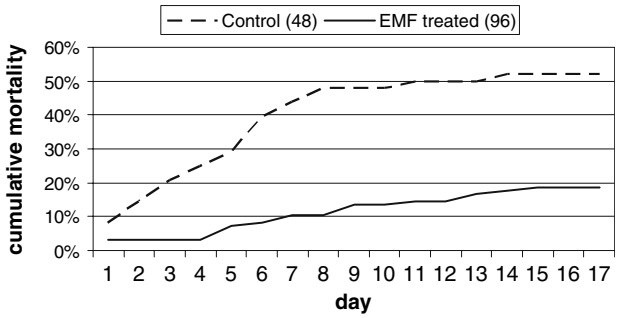

Fig. 4 Experiment 1: Mortality 144 goldfish with infections

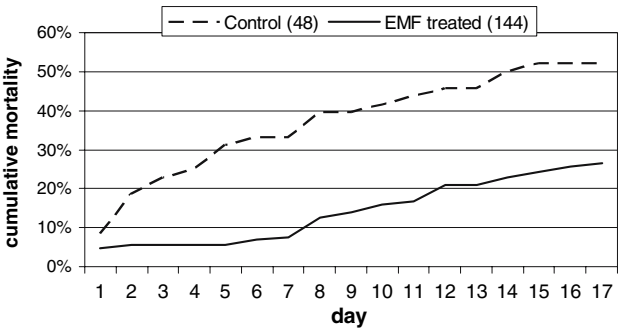

Fig. 5 Experiment 2: Mortality 192 goldfish with infections

In order to find out more about the field strength dependency of the effect and to determine the required distance between tubs for parameter variation experiments, a next experiment was done with varying field strength.

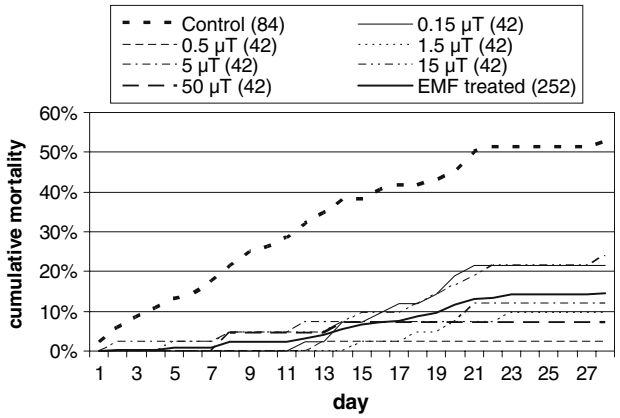

Fig. 6 Experiment 3: Mortality 336 goldfish with infections

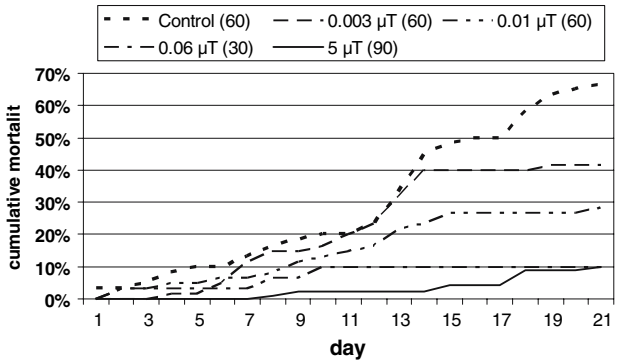

Fig. 7 Experiment 4: Mortality 300 goldfish with infections

There were four control groups and two test groups for six different field strengths varying from 0.15 to 50 μT. Each group contained 21 fish initially.

In Fig. 6 data for six different field strength, ranging from 0.15 μT (thin solid line) to 50 μT (thick, long dashed line) the mortality development is given as before. Again, the control groups suffered mortality increasing to 52% on day 28 (upper, thick short dashed line). The average of all treatment groups is also given (thick solid line), this increases to 15% at day 28. The end values were 52 ± 29% untreated and 15 ± 11% treated (all together). Again, the treatment resulted in a strong decrease in mortality at all field strength levels.

The result indicates that the decrease in mortality after EMF exposure was equally strong between 0.15 and 50 μT, except at 15 μT. There seems to be a decrease in effectiveness from 0.05 μT downwards which becomes noticeable at 0.015 μT.

Since for our application especially the lowest field strength range is important, a follow up experiment was performed that realized lower field strength than 0.15 μT by placing test tubs in the stray field of a 5 μT tub. This was done because the voltages and currents needed to create such small fields are so low that electronic noise became too high. The 5 μT tub is built as a solenoid coil along the complete height of the tub. Such a solenoid coil has a small but rather uniform stray field that is suitable for the analysis that we wished to perform.

The result is given in Fig. 7. From the .06 μT group in Fig. 7 one replicate was lost due to a technical problem.

It can be observed that over the whole period mortality is highest in the control group and lowest in the group treated at 5 μT. From day 7 onwards mortality is strictly increasing with decreasing field strength.

Even at 0.003 μT (3 nT) these results indicate that there still is an effect of the treatment.

These results indicate that in order to be reasonably confident that there is no influence of EMF on control groups one must go to field strength levels of 1 nT or lower.

4.3 In vitro experiments chicken broilers

Coccidial lesion of intestines due to *Eimeria acervulina* and *Eimeria maxima* were significantly lower in the positive EMF group compared to the positive control group on day 21 and 29 as presented in Fig. 8:

The results of the feed conversion are shown in Fig. 9. Feed conversion of the broilers of the EM group with infection (as in practice) was significantly lower than controls for both experiments.

A significant and economically highly relevant improvement in feed conversion is achieved by EMF treatment under Coccidiosis infection. In chickens, one explanation for less feed uptake indicates could be less energy spent on developing infections.

Moreover it should be noted that the reduction in feed conversion achieved rivals the best results achieved in comparable trials with preventive antibiotics (now illegal in animal feed in the EU). This indicates that EM treatment is as effective in suppressing infections and the resulting productive loss in chicken as preventive antibiotics were.

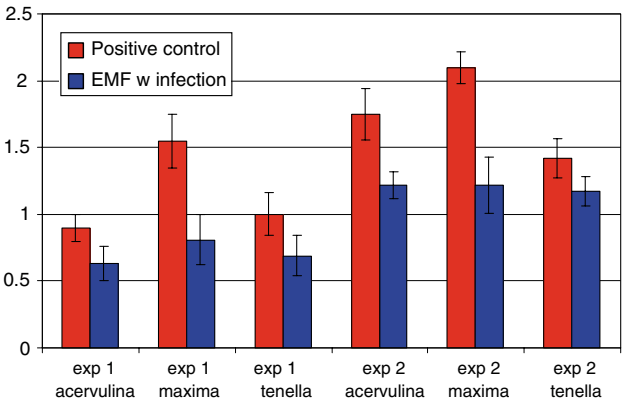

Fig. 8 Intestine lesions (blind score)

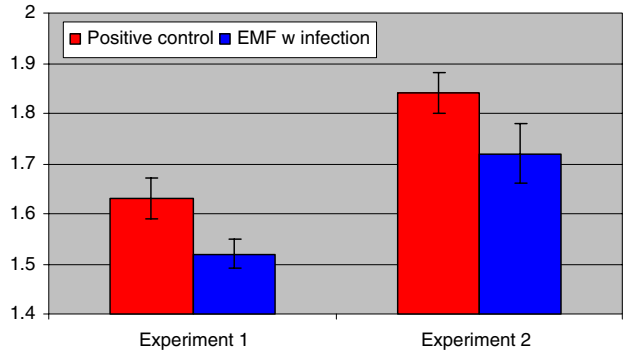

Fig. 9 Feed conversion (kg growth/kg feed)

5 Conclusions

This research indicates that the ELF EMF treatment with the Immunent signal is capable of stimulating the immune system. On a cell level phagocyting cells have been shown to become more active after treatment. This result was obtained at 1.5 mT as well as at 5 μT.

On the level of the whole organism it was shown that at and around 5 μT the survival of diseased fish could be greatly improved by the treatment. In the light of the cell experiments also reported on, it is likely that this is based on an increased immune activity of the fish. The effect is present in a large window of field strengths around 5 μT, and one must go to the level of 1 nT or lower to be confident that the effect is no longer present. Proper distances between controls and test tubs are therefore important.

LF EMF treatment reduces the damage, in terms of intestinal lesions of Coccidiosis infection in broilers. Moreover LF EMF treatment was shown to improve feed conversion up to 12 points, equivqlent to some 8% reduced feed uptake for equal growth. These results support the hypothesis that LF EMF improves the immune competence of the animals.

Because of the low field strengths required, and the surprisingly large effects on animal health, the results indicate that practical application with important economic advantages for farmers is possible. Moreover, the new experimental model used in this study can be important for bioeffects studies for EMF in general.

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