Effects of sublethal exposure of European brown hares to paraoxon on the course of tularemia

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Abstract

OBJECTIVES: The causative agent of tularemia *Francisella tularensis* is highly infectious and lagomorphs are important reservoirs and a source of human disease. The aim of the present study was to test the hypothesis that sublethal exposure to pesticides increases the susceptibility of hares to *F. tularensis* and modulates the course of the infection.
METHODS: Experimental hares were allocated to a) control, b) paraoxon-treated, c) *F. tularensis*-treated, and d) paraoxon-and-*F. tularensis*-treated groups of five specimens on a random basis and subcutaneously inoculated with a wild *F. tularensis* subsp. holarctica strain (a single dose of 9×10^8 CFU pro toto) and/or injected a sublethal dose of paraoxon (100 μg/kg). Group differences were evaluated using survival curves, oxidative stress responses as well as caspase-3 and acetylcholinesterase activities in whole blood samples collected on day 2 post exposure.
RESULTS: The paraoxon-and-*F. tularensis*-treated group showed a rapid onset of clinical signs and all deaths occurred on days 2 and 3 post exposure. *F. tularensis*-inoculated hares survived from 3 to 10 days, while only one hare died on day 12 in the paraoxon-treated group. Survival curves in the three exposed groups were significantly different from the control and median survival in *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-treated hares amounted to 7 and 2 days, respectively. Compared with controls, significant responses included an eight- and seven-fold activation of caspase-3 in *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-treated hares, respectively, and a 1.5-fold decrease of blood acetylcholinesterase activities in the paraoxon-treated and paraoxon-and-*F. tularensis*-treated groups. There was a 1.3- to 1.4-fold decrease of the ferric reducing antioxidant power in blood of *F. tularensis*-inoculated hares and the paraoxon-and-*F. tularensis*-treated group, respectively. The blood lipid peroxidation levels were of no differences among the four experimental groups.

ORIGINAL ARTICLE

CONCLUSIONS: Results of this study can help understand the pathogenesis of tularemia and mortality of hares in agricultural habitats. Use of anticholinesterase agents in agriculture can pose a threat of infectious disease outbreaks and higher mortality in wildlife populations.

Abbreviations:

ACHE	- acetylcholinesterase
C	- controls
CFU	- colony-forming units
FRAP	- ferric reducing antioxidant power
IFN	- interferon
LD_{50}	- median lethal dose
LSD test	- the least significant difference test
P	- paraoxon-treated hares
T	- *F. tularensis*-inoculated hares,
TBARS	- thiobarbituric acid reactive substances
TNF	- tumour necrosis factor
TP	- paraoxon-and- *F. tularensis*-treated hares

INTRODUCTION

Francisella tularensis belongs among the most infectious and pathogenic bacteria (Pechous *et al.* 2009). Although it is able to induce tularemia in a wide variety of organisms, human infections are frequently associated with contact with lagomorphs such as the European brown hare (Gyuranecz *et al.* 2010, Mueller *et al.* 2007, Pikula *et al.* 2004a,b, Pohanka *et al.* 2007, Treml *et al.* 2007). Interestingly, hares have been demonstrated recently (Bandouchova *et al.* 2011) to survive a high single dose of 2.6×10^9 bacterial colony-forming units (CFU) administered subcutaneously. These results, showing low susceptibility of European brown hares to tularemia, contrast with some other reports classifying hares as highly susceptible (Krivinka 1939, Olsufjev & Dunajeva 1970). Considering the public health importance of this host, the circumstances leading to the development of clinical tularemia in hares under natural conditions should be further studied.

Epizootics of infectious diseases in wildlife have been found to be more severe in areas contaminated by environmental pollutants and the possibility of population level effects associated with contaminant-induced immunosuppression has been demonstrated (Grasman 2002). Synergistic effects between pesticides and natural stressors remain to be challenging issues (Relyea & Hoverman 2006) as well as the chronic low-level exposure to chemical mixtures (Sanderson and Solomon 2009) with additive or joint independent actions (Kortenkamp *et al.* 2007). The wide agricultural use of organophosphate-based pesticides makes exposure of humans and animals unavoidable and may result in both acute effects and chronic damage to the nervous system (Stephens *et al.* 1995). Organophosphates as cholinesterase inhibitors impair the breakdown of the neurotransmitter acetylcholine (Pohanka *et al.* 2010a). Acetylcholine has immunosuppressive effects as it significantly and concentration-dependently decreases endotoxin-inducible pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukins. An insufficient neuroimmunomodulatory function mediated through the cholinergic anti-inflammatory pathway with nicotinic acetylcholine receptors on macrophages may result in increased susceptibility to infection (Pavlov *et al.* 2003, Pohanka *et al.* 2011).

Considering the pathogenesis of tularemia and host protection, the cytokine response may be part of a local barrier function of the skin (Stenmark *et al.* 1999), while TNF alpha and interferon (IFN) gamma are essential for control of infection, as depletion of either converts typically sublethal infections into lethal ones (Pechous *et al.* 2009).

The aim of the present study, therefore, was to test the hypothesis that sublethal exposure to an anticholinesterase pesticide paraoxon increases the susceptibility of hares to *F. tularensis* and modulates the course of the infection including mortality. For this purpose we compared the effects of single and combined exposures to paraoxon and *F. tularensis* and evaluated survival curves, oxidative stress responses, caspase-3 and acetylcholinesterase activities.

MATERIAL AND METHODS

Experimental animals

One-year-old European brown hare males (*Lepus europaeus*), a total number of twenty, purchased from the Hare Breeders´ Association of the Czech Republic were used for the study. They were fed standard granules for rabbits without anticoccidials and high quality hay. Drinking water was provided *ad libitum*. Prior to the experiment the hares appeared healthy, in an excellent nutritional state, and were certified free of tularemia and brucellosis based on tube agglutination tests. Experiments were performed in the Biosecurity level 3 laboratory of the Department of Infectious Diseases and Microbiology (University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic).

Experimental microorganism

A wild strain of *Francisella tularensis* isolated from a European brown hare from South Moravia in 2004 was used for experimental infections in this study. This isolate, subtyped as *Francisella tularensis* subsp. *holarctica* by proteomic techniques, has already been used in a previous experimental study on the susceptibility of hares (Bandouchova *et al.* 2011) and selected murine and microtine species (Bandouchova *et al.* 2009a, 2009b). Experimental infections were performed using a suspension of *F. tularensis* cells harvested from a culture growing on blood agar supplemented with L-cysteine using sterile physiological saline solution. After thorough mixing we measured the absorbance of the suspension at 605 nm using a spectrophotometer (Unicam Helios Gamma&Delta, Spectronic Unicam, United Kingdom) in order to determine the

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number of bacterial cells per unit volume according to McFarland´s standard (Murray *et al.* 2003). The number obtained was only approximate and was used to estimate the dilution necessary to achieve the dose required. The exact infectious dose was then determined by plating ten-fold serial dilutions and counting CFU in the suspension administered to experimental animals. Colonies were counted after 72 h of incubation at 37 °C. Virulence of the *F. tularensis* strain was tested by inoculation of BALB/c mice.

Experimental design

Experiments were performed in compliance with laws for the protection of animals against cruelty and were approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Experimental hares were allocated to the control, paraoxon-treated, *F. tularensis*-treated, and paraoxon-and-*F. tularensis*-treated groups (five specimens each) on a random basis. Hares from the *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-inoculated groups were administered subcutaneously with a single dose of 9×10^8 CFU of *F. tularensis pro toto* into the dorsal trunk area, while sublethal doses of paraoxon (100 µg/kg; Paraoxon-ethyl, PESTANAL®, analytical standard, Fluka Analytical, Sigma-Aldrich, St. Louis, USA; 0.1 ml of propylenglycol was used for dilution of paraoxon, Propylenglycolum, Tamda, a.s., Olomouc, Czech Republic) were injected subcutaneously to each hare in the paraoxon-treated and paraoxon-and-*F. tularensis*-treated groups. Animals were examined every 6 hours for developing signs of infection. Data on days (hours) elapsed from inoculation to death of experimental animals were then recorded and used to compare survival curves.

Blood was collected from the jugular vein using a heparinised set Omnican® 40 (Braun, Germany) every other day from days 0 to 22 when the surviving hares were euthanised. Samples of whole blood were frozen immediately after collection (–80 °C). Necropsy was performed in hares that died or were euthanized. Whole blood samples were employed for biochemical measurements of caspase and acetylcholinesterase activities, ferric reducing antioxidant power and lipid peroxidation. Blood samples were also examined for the presence of *F. tularensis* by culture and the mouse inoculation test.

Biochemical assays

ACHE activities were assessed using the modified Ellman´s method as described previously (Pohanka *et al.* 2008). Caspase-3 activity was assayed by a commercially available colorimetric kit (Sigma-Aldrich, Saint Louis, Missouri, USA) as recommended by the producer. The optical density was compared with p-nitroaniline in order to construct the calibration curve. The ferric reducing antioxidant power (FRAP) for estimation of low molecular weight antioxidants and thiobarbituric acid reactive substances (TBARS) for assay of malondialdehyde representing lipid peroxidation were assayed as described earlier (Pohanka *et al.* 2010b). The total protein kit (Sigma-Aldrich, Saint Louis, Missouri, USA) was used to assay the concentration of proteins in samples in a way recommended by the producer. Results of the above mentioned markers were then related to the calculated total protein in blood.

Statistical analysis

Statistica for Windows® 7.0 (StatSoft, Tulsa, OK, USA) was used to compare different treatment groups via one-way analysis of variance (ANOVA) and *post-hoc* analysis of means via the LSD test. The homogeneity of variances was tested by Levene´s test. Non-homogenous parameters, as determined by Levene´s test, were log-transformed prior to analysis. In these cases, the non-parametric Kruskall-Wallis test was used for the comparison of treatment groups. GraphPad Prism 4 (GraphPad Software, San Diego, USA) was also used for statistical analyses and graphing data. Survival curves in experimental animals were compared using the logrank test and the calculation of two-tailed *p*-values. Values of $p<0.05$ and $p<0.01$ were considered statistically significant and highly significant, respectively, for all tests.

RESULTS

There was no mortality in the control group and the hares showed no clinical signs of disease during the experiment. The paraoxon-and-*F. tularensis*-treated group showed a rapid onset of clinical signs such as fever as high as 41 °C (normal rectal temperature ranged from 38.4 to 38.9 °C in control hares), lethargy and anorexia and all deaths occurred on days 2 and 3 post exposure. *F. tularensis*-inoculated hares survived from 3 to 10 days, while only one hare died on day 12 in the paraoxon-treated group. Survival curves in the three exposed groups were significantly different from the control (logrank test, chi square = 12.61, df = 2,

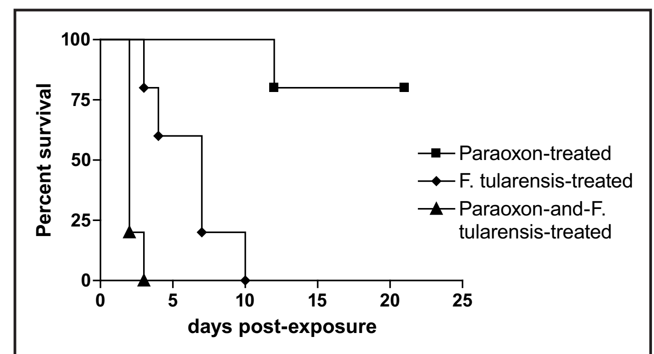

Fig. 1. Survival curves in European brown hare groups after single and combined exposures to the acetylcholinesterase inhibitor paraoxon and *Francisella tularensis* infection. No mortality occurred in the control group (data are therefore not shown).

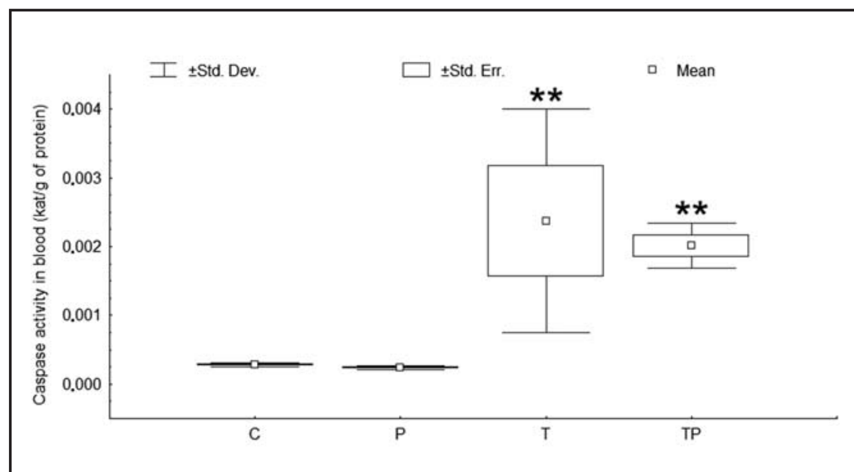

Fig. 2. Differences in blood caspase-3 activities in three treatment groups of hares and the control on day 2 post exposure. C = controls, P = paraoxon-treated hares, T = *F. tularensis*-inoculated hares, TP = paraoxon-and- *F. tularensis*-treated hares, $^{**}p<0.01$ compared with the control (n=5 in each group).

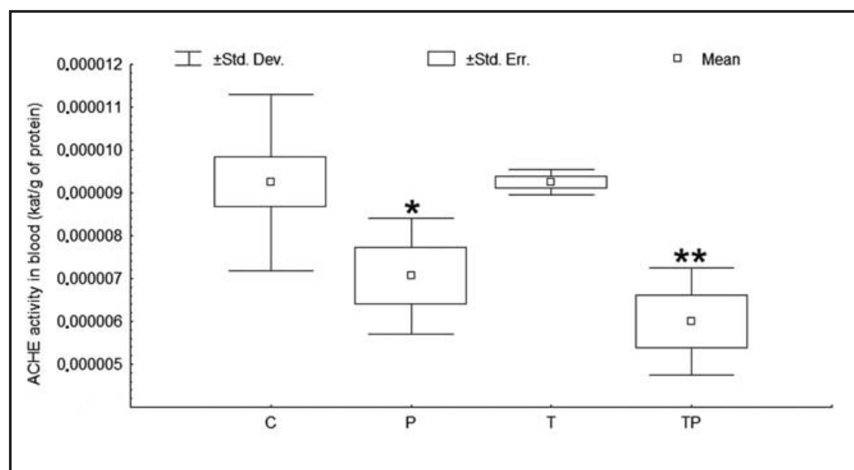

Fig. 3. Differences in blood acetylcholinesterase (ACHE) activities in three treatment groups of hares and the control on day 2 post exposure. C = controls, P = paraoxon-treated hares, T = *F. tularensis*-inoculated hares, TP = paraoxon-and- *F. tularensis*-treated hares, $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control (n=5 in each group).

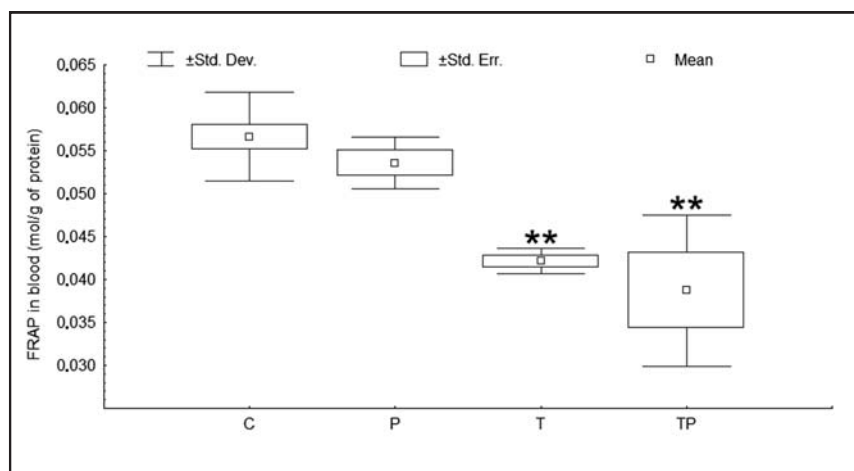

Fig. 4. Differences in blood ferric reducing antioxidant power (FRAP) in three treatment groups of hares and the control on day 2 post exposure. C = controls, P = paraoxon-treated hares, T = *F. tularensis*-inoculated hares, TP = paraoxon-and-*F. tularensis*-treated hares, $^{**}p<0.01$ compared with the control (n=5 in each group).

$p=0.0018$, cf. Figure 1). Median survival in *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-treated hares amounted to 7 and 2 days, respectively.

Group differences were also evaluated using whole blood oxidative stress responses as well as caspase-3 and acetylcholinesterase activities. Figures 2 and 3 show differences in caspase-3 and acetylcholinesterase activities among three treatment groups of hares and the control, while Figure 4 demonstrates differences in ferric reducing antioxidant power. Interestingly, the blood caspase-3 activity was nearly the same in control and paraoxon-treated hares, and its activation resulted in an eight- and seven-fold increase in *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-treated hares on day 2 post exposure, respectively. Blood acetylcholinesterase activities in paraoxon-treated and paraoxon-and-*F. tularensis*-treated hares were 1.5 times lower than in the control two days after exposure. The FRAP levels in blood were 1.3 to 1.4 times decreased both in *F. tularensis*-inoculated hares and the paraoxon-and-*F. tularensis*-treated group on day 2 post exposure. There were, however, no significant differences in blood TBARS levels among the four experimental groups of hares on day 2 of the experiment.

DISCUSSION

Results of this experimental study support our hypothesis that European brown hares exposed to an acetylcholinesterase inhibitor are more susceptible to infection by a virulent *Francisella tularensis* subsp. *holarctica* strain. While the single exposure of hares to paraoxon at the selected dose of 100 μg/kg proved mainly sublethal and only one hare died 12 days post exposure, hares inoculated subcutaneously with a single infectious dose of 9×10^8 CFU of *F. tularensis pro toto* survived from 3 to 10 days. On the other hand, hares exposed to the combination of the above toxic and infectious doses succumbed within 2 to 3 days. The median sur-

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vival in *F. tularensis*-inoculated hares was significantly higher than in paraoxon-and-*F. tularensis*-treated hares. It is, therefore, clear that exposure of hares to an anticholinesterase agent significantly modulates the course of the *F. tularensis* infection.

To the best of our knowledge, there are no published data on the LD_{50} of paraoxon in European brown hares. The subcutaneous dose of 100 μg/kg employed in this study was, therefore, derived as approximately one fourth of the LD_{50} in rats (Krutak-Krol & Domino 1985) or one half of the LD_{50} in rabbits (Maxwell *et al.* 2006) and excepting one hare resulted in a sublethal exposure. The infectious dose of *F. tularensis* administered to infect hares by tularemia in this study (i.e. 9×10^8 CFU of *F. tularensis*) was very high. However, European brown hares proved less susceptible than expected in the previous experiment (Bandouchova *et al.* 2011).

Owing to the quick development of mortality in paraoxon-and-*F. tularensis*-treated hares, sufficient data for statistical comparisons of all groups were only obtained from blood samplings on day 2 of exposure. Results on oxidative stress responses and changes in caspase-3 and acetylcholinesterase activities are therefore based on this sampling.

Apoptosis of mammalian cells is usually mediated through activation of the caspase cascade in which caspase-3 has a key effector role (Kothakota *et al.* 1997). It is known that organophosphates induce apoptosis through exposure to low or high concentrations of organophosphates, independently of ACHE inhibition. For example, paraoxon has been found to induce apoptosis in cultured neurons via a caspase-9-dependent pathway leading to caspase-3 activation (Wu *et al.* 2005). As shown in the present study, however, activation of caspase-3 in whole blood of experimental hares was a characteristic of tularemia. This finding corresponds with data on induction of extensive caspase-3 activation and apoptotic cell death in the tissues of hosts infected by *F. tularensis* (Wickstrum *et al.* 2009).

Paraoxon, the active metabolite of parathion, can be considered one of the most potent acetylcholinesterase-inhibiting insecticides that can decrease acetylcholinesterase up to 70% of inhibition elicited by the nerve agent sarin (Kalra *et al.* 2002, Pohanka *et al.* 2010a). In general, a reduction in activity to lower than 20 to 50 % of normal values results in clinical signs and mortality (Poppenga 2007). As demonstrated in the present study, the sublethal exposure reduced significantly blood acetylcholinesterase activities both in paraoxon-treated and paraoxon-and-*F. tularensis*-treated hares within two days of exposure. Excepting one hare, however, the reduction was not sufficient to cause mortality in the single paraoxon-exposure group.

The host defence against various intracellular pathogens including *F. tularensis* involves a non-specific response of reactive nitrogen and oxygen species production that can induce oxidative stress (Lindgren *et al.* 2004). Likewise, organophosphorus compound toxicity may be associated with oxidative status changes in the organism (Pohanka *et al.* 2009a). These processes can result in consumption of antioxidants as well as peroxidation of cellular lipids due to hydroxyl radical production. It is possible to evaluate the ferric reducing antioxidant power (FRAP) and lipid peroxidation (TBARS) as a measure of the total antioxidant capacity and oxidative damage, respectively. Non-enzymatic antioxidants such as ascorbic acid, uric acid, bilirubin, vitamin E, α-tocopherol and albumin contribute to the ferric reducing antioxidant power and the reaction is linearly related to their molar concentrations (Benzie and Strain 1996). Changes of low-molecular-weight antioxidants in *Francisella tularensis* infected hosts have already been described in laboratory mice and common voles (Pohanka *et al.* 2009b). While blood levels of FRAP were decreased both in the *F. tularensis*-inoculated and paraoxon-and-*F. tularensis*-treated groups, no significant differences in blood TBARS levels among the four experimental groups of hares were noticed on day 2 of the experiment. The data demonstrate that the antioxidant system got quickly depleted in tularemic hares. On the other hand, two days of exposure were not long enough for lipid peroxidation to develop because increased blood TBARS levels were shown not earlier than on day 4 in hares infected by *F. tularensis* (Bandouchova *et al.* 2011). Interestingly, the course of tularemia as well as oxidative stress may be modulated by oximes, i.e. specific antidotes used for acetylcholinesterase reactivation in patients intoxicated by organophosphorus compounds (Pohanka *et al.* 2010b,c).

Regarding the wide use of organophosphate-based pesticides and geographic distribution of natural foci of tularemia (Pikula *et al.* 2003, Pikula *et al.* 2004a,b, Stephens *et al.* 1995), the model of combined exposure of experimental hares to individual stressors is ecologically realistic. Experimental hares were infected by the subcutaneous route, which is clinically relevant because it imitates one of the natural routes of tularemia transmission via ticks that carry the agent. It was demonstrated that the numbers of *F. tularensis* cells fluctuate from 40 to 69 300 in infected ticks from natural foci of tularemia (Hubalek *et al.* 1996). In light of this, however, fatal infection due to single exposure of hares to *F. tularensis* in this way would require a really heavy tick infestation. On the other hand, determination of the minimum infectious dose of *F. tularensis* in combination with exposure to organophosphates would be an interesting issue for further studies.

When evaluating wildlife toxicity, it is necessary to distinguish bacterial haemorrhagic septicaemia, disseminated intravascular coagulation and anticoagulant poisoning (Beklova *et al.* 2007, Krizkova *et al.* 2007, Pikula *et al.* 2007). Interestingly, the field study from Spain documented an interaction between the use of anticoagulant rodenticides to control the common vole (*Microtus arvalis*) population peak and tularemia char-

acteristics such as prevalence and spread (Vidal *et al.* 2009). Traditional toxicological studies focus on single stressor toxicity in single species with standard endpoints such as the lethal dose of a substance resulting in 50% mortality. Experiments employing combined exposures to multiple stressors can, however, provide more environmentally relevant data (Pikula *et al.* 2010). Further studies are necessary to explain the exact pathogenesis of enhancement of the microbial infection by the action of an acetylcholinesterase inhibiting agent. The dysregulated production of cytokines by the cholinergic anti-inflammatory pathway provides, however, a feasible explanation to the increased susceptibility of hosts to infection (Pavlov *et al.* 2003, Pohanka *et al.* 2011).

CONCLUSION

Synergistic effects between acetylcholinesterase inhibitors and the infectious agent *Francisella tularensis* can increase the susceptibility of the host and may enhance the overall wildlife mortality. Bringing together new pieces of knowledge on health effects of low concentrations of an anticholinesterase agent, it can help us better understand the mechanisms of action and the effects of pesticides released by humans and infectious agents in wild animal populations. Further studies into these important issues will be, however, necessary.

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