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The impact of carbofuran on acetylcholinesterase activity in *Anisakis simplex* larvae from Baltic herring

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Abstract

The aim of this study was to investigate the impact of carbofuran, a carbamate pesticide on the enzymatic activity of acetylcholinesterase (AChE) in the larvae of the herring nematode parasite *Anisakis simplex*. *A. simplex* larvae collected from herring were exposed to carbofuran *in vivo* at concentrations of 50, 100, 500 and 1000 μg/l, for 24, 48 and 72 h, at a temperature of 4 °C. Generalized Linear Models (GLM) were applied to analyze the relationship between AChE activity and carbofuran concentration, the time of exposure and the biological parameters of the host. The results indicate that *A. simplex* larvae have a high threshold of sensitivity to carbofuran. The average enzymatic activity was higher in parasites obtained from male hosts, when compared with female hosts. These data suggest that host sex-dependent biological processes may also influence AChE enzymatic activity in parasites.

Keywords: AChE; *Anisakis simplex*; Baltic sea; Carbamate pesticide; Carbofuran

1. Introduction

Carbofuran is a carbamate pesticide that has wide applications in agriculture as an insecticide. It is well known that carbamate pesticides inhibit acetylcholinesterase (AChE) activity, which consequently leads to accumulation of acetylcholine in synapses and continuous stimulation of the neural system. Measurements of AChE are commonly used as a biomarker of exposure to different contaminants, including pesticides [1–5].

The main role of AChE in vertebrates is regulation of acetylcholine levels by the rapid hydrolysis of acetylcholine into the inactive products: choline and acetic acid. Acetylcholinesterase has been also defined as a key enzyme in neuromuscular transmission in many nematode species. Neuromuscular AChEs exist in multiple molecular forms in free-living and parasitic nematodes. In contrast to vertebrates, distinct forms of AChE are encoded by separate genes in nematodes. The free-living nematode *Caenorhabditis elegans* has been shown to possess three separate genes that encode the kinetically distinct AChE classes A, B and C [6]. The three major classes of AChE have also been characterized in the plant parasitic nematodes *Heterodera glycines* and *Meloidogyne* [7,8]. Class C of the AChEs from *Meloidogyne* are highly resistant to carbamates and organophosphates [8]. The available data indicate that there is restricted expression of somatic AChE in some parasitic nematode species, a single AChE form has been identified in *Nippostrongylus brasiliensis* [9] and *Parascaris equorum* [10], whereas two forms of AChE have been detected in *Trichinella spiralis* [11].

In addition to the membrane-bound (neuromuscular) enzymes, many parasitic nematodes, in particular those that colonize the alimentary tract of their vertebrate hosts, synthesize AChEs in specialized secretory glands and release it into their external environment [12]. Secreted variants of the enzyme have been described in *N. brasiliensis* [13] and *Dictyocaulus viviparus* [14], but the roles of these

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secreted forms are still under discussion. One of the suggested functions of the secreted forms of parasite AChE is modulation of the host's immune response and inhibition of secretory responses [15,16].

Previous studies carried out by Podolska and Napierska [17] indicated that there was a significant negative correlation between the host and parasite AChE activity. AChE inhibition in muscle tissue of herring caught in contaminated areas was accompanied by very high enzymatic activity in the herring parasites, *A. simplex* larvae. The authors suggested that in contaminated areas, AChE inhibition in herring may lead to the accumulation of acetylcholine. This host biochemical response may be adverse for parasite survival, therefore, the nematodes may produce a large amount of AChE in order to inactivate the host's acetylcholine. Alternatively, the high level of AChE in parasites may be the result of adaptive mechanisms to protect the organism against pesticides and organophosphates. AChE insensitivity to inhibitors (organophosphates and carbamates) has previously been reported in insects [18–20]. Fournier et al. [21] suggested that resistance to organophosphates in *Drosophila melanogaster* could result from AChE overproduction. However, there are no data suggesting how pesticides interfere with the AChE of internal fish parasites. Thus, the aim of this study was to investigate the impact of carbofuran, a carbamate pesticide, on the activity of acetylcholinesterase from the larvae of the herring nematode parasite *A. simplex*.

2. Materials and methods

2.1. Biological material

Live *A. simplex* larvae were collected from the herring *Clupea harengus* caught from the middle coast region of the Baltic (Rowy) over a period of time from 24th April to 11th May 2006. The middle coast area is characterized by a low contaminant input and has been regarded as "a reference region" in previous studies that investigated the biological effects of contaminants [22,23]. Basic biological parameters (body length, weight, age, sex and gonad developmental stage) were recorded for each herring. *A. simplex* larvae (>500 individuals) were collected separately from male and female hosts and allocated to experimental groups (pooled samples of 4–7 larvae from the same individual herring in each group). Next, the parasites were placed in eppendorf tubes and exposed *in vivo* to carbofuran (PESTANAL®, analytical standard, Riedel-de Haën, synonym: 2,3-dihydro-2,2-dimethyl-7-benzofuranol *N*-methylcarbamate) at a concentration of either 50, 100, 500 and 1000 μg/l for periods of either 24, 48 and 72 h, at a temperature of 4 °C. Carbofuran was dissolved in a physiological NaCl solution. The exposure was conducted in the dark to avoid photodegradation of carbofuran. Larvae in physiological NaCl solution without pesticide were used as a control in the experiment. After exposure to carbofuran, *A. simplex* larvae were immediately frozen at −80 °C for biochemical analyses.

2.2. Preparation of tissue homogenates

AChE extraction was performed on 10–30 mg of *A. simplex* larvae tissue (108 pooled samples) using 0.02 M phosphate buffer (pH 7.0) containing 0.1% Triton X-100. The worms were homogenized in 10 volumes of buffer (10 ml buffer per g wet weight tissue) and centrifuged at 10,000*g* for 20 min at 4 °C. An aliquot of the supernatant was stored at −80 °C and used in an assay to determine AChE activity.

2.3. Determination of enzyme activity

Determination of AChE activity was performed using a method described by Ellman et al. [24] and adapted for use with a microplate reader [25]. The enzyme kinetics were monitored at 412 nm for 3 min. The standard reaction mixture, with a final volume 0.380 ml, contained 0.02 M phosphate buffer, pH 7.0, 0.5 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) and 2.6 mM ACTC (acetylthiocholine chloride). Protein concentration was determined as described by Bradford [26], using a Protein Kit II from Bio-Rad laboratories and bovine serum albumin (BSA) as the protein standard.

3. Statistical analyses

Generalized linear models (GLM) [27] were applied to analyze the relationship between enzymatic activity and carbofuran concentration, the time of exposure and biological parameters of the host. The following model was used to fit the data:

$$\text{AChE activity} = \text{CC} + \text{t} + \text{LT} + \text{sex} + \text{gon}$$

where CC, concentration of carbofuran; t, time of exposure; LT, the total length of herring; sex, the sex of fish; gon, the gonad maturity stage. First, the initial model (which included all of the considered variables and factors) was fitted. Corner point parameterization was used, i.e. the factor effects for level one were assumed to be zero for all factors. Thus, the factor effects for other levels can be regarded as the differences between the effect at a given level and the effect at level one. Body length was taken to be covariate, and the other terms (CC, t, sex and gon) were treated as factors in the analysis. The error was assumed to be normal and identity link function was used. Next, the significance of factors and covariates was tested and only the significant terms were left in the final model. Similarly, factor levels that did not produce a significantly different response in enzymatic activity were grouped into new factor levels. The tests were performed by deletion, i.e. only those terms whose deletion did not result in a significant increase in deviance (the GLM measure of discrepancy between modeled and observed values) were left in the