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Safety of the anthelminthic drugs levamisole, fenbendazole, and ivermectin administered in therapeutic baths for the common carp *Cyprinus carpio*

Jitka Kolarova, Alzbeta Stara, Eliska Zuskova, Josef Velisek*

South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

**Corresponding author: kolarova@frov.jcu.cz*

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Abstract: Due to the safe "off label" use of selected antiparasitic drugs in fish, we assessed the effects of a therapeutic bath with levamisole (50 mg/l), fenbendazole (25 mg/l), and ivermectin (0.031 mg/l) on the haematological and biochemical blood indices, oxidative and antioxidant variables, and gill histology of the common carp (*Cyprinus carpio*). Levamisole did not affect the haematological profile, but significantly increased ($P < 0.01$) the plasma glucose, lactate, and ammonia concentrations, alkaline phosphatase, and alanine aminotransferase activities, TBARS (muscle, liver), total superoxide dismutase activity (muscle), and catalase activity (liver) and significantly decreased ($P < 0.01$) plasma aspartate aminotransferase and glutathione reductase activity (gill, liver, muscle). Ivermectin led to a significantly ($P < 0.01$) greater muscle total superoxide dismutase activity compared to the controls, whereas the haematological and biochemical indices remained unchanged. On the other hand, fenbendazole did not affect the haematological or biochemical indices, and the oxidative stress parameters and antioxidant indices did not differ from the controls. The bath in FBZ can be recommended for safe antiparasitic treatment in carp.

Keywords: blood biochemistry; haematology; histology; oxidative stress

Flatworms of the class Monogenea are common ectoparasites of freshwater and marine fish, with many species infecting cyprinids (Kuchta et al. 2020), which impacts intensive carp farming in Central Europe. Species of *Dactylogyrus* and *Gyrodactylus* damage the gill epithelium and skin, which may lead to significant economic losses. Recommended drugs for fish ectoparasite treatment include fenbendazole, levamisole, and ivermectin (Noga 2010; Alves et al. 2019). Reduced food intake in the fish necessitates treatment via therapeutic baths. No veterinary medicinal product (VMP) containing these substances [the maximum residue limit (MRL) must be established] is currently authorised for fish intended for human consumption. Veterinarians must, in accordance with Decree 344/2008 Coll., prescribe and use drugs according to the principle of the medical product

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cascade of choice. Veterinary medicinal products containing the active substances fenbendazole, levamisole, and ivermectin registered for species intended for human consumption, other than fish, are available. Using the drug selection cascade for off-label use, it is permissible to administer them to fish intended for consumption, provided that the specified withdrawal period of 500 degree-days is followed.

Levamisole hydrochloride (LHC) is an active substance in VMPs authorised for pigs and poultry and is indicated for the treatment of intestinal tract parasitosis. A common recommended treatment concentration for a therapeutic bath of fish is 50 mg/l for two hours (Stoskopf 1993; Kibenge et al. 2021).

Fenbendazole (FBZ) is an active substance in VMPs authorised for use in cattle, sheep, goats, and pigs. For the treatment of fish, a concentration of 25 mg/l for 12 h is recommended (Noga 2010). Repeated oral administration of FBZ reduces *Dactylogyrus* sp. infections of *Labeo rohita* (Gupta et al. 2020) and tapeworm infections in the common carp.

Ivermectin (IVM) is an active substance in VMPs authorised for cattle, sheep, pigs, and horses. For the treatment of monogenean infections, a 12-h bath at 0.031 mg/l is recommended (Santamarina et al. 1991). The oral administration of IVM (0.2 mg/kg) in Atlantic salmon (*Salmo salar*) against sea lice (*Lepeophtheirus salmonis*) reduced all the parasite stages (Pike and Wadsworth 2000). However, IVM shows environmental toxicity, being rapidly and widely distributed throughout ecosystems, bioaccumulates at high levels, and persists as long-term residue (Wang et al. 2020).

This study aimed to assess the safety of FBZ, LHC and IVM to the common carp *Cyprinus carpio* in therapeutic baths at concentrations recommended for off-label use in fish as indicated by the haematological and biochemical profiles, oxidative stress response, antioxidant biomarkers, and gill histology.

MATERIAL AND METHODS

Veterinary medicine products

The VMP containing levamisole hydrochloride 800 mg/g plv was purchased from Kela Laboratoria NV (Hoogstraten, Belgium). The VMP containing FBZ (25 mg/ml), Panacur 25% p.o. susp., was purchased from Intervet International B.V. (Boxmeer, The Netherlands). The VMP containing IVM (10 mg/ml), Biomectin inj., was purchased from Vetoquinol s.r.o. (Prague, Czech Republic).

Fish

Juvenile common carp *C. carpio*, 48 fish in total, with a mean total length of 106.8 ± 13.00 mm and a body weight of 22.5 ± 8.07 g, were obtained from the breeding facility of the Faculty of Fisheries and Protection of Waters at the University of South Bohemia, Czech Republic.

All the laboratory procedures were conducted in compliance with Czech Republic regulations 166/1996 and 246/1992 and approved by the Departmental Expert Committee for Authorisation of Experimental Projects of the Ministry of Education, Youth, and Sports of the Czech Republic (permit MSMT 4394/2017-2).

Experimental design

The fish were randomly separated into four groups of six as follows: control (C) in tap water only; LHC 50 mg/l for 2 h; FBZ 25 mg/l for 12 h, IVM 0.031 mg/l for 12 hours. The conditions were duplicated twice for a total of eight groups, each held in 300 l glass aquarium containing medication dissolved in tap water.

The hydro-chemical parameters of tap water were: temperature 21.5 ± 0.5 °C; acid neutralisation capacity $ANC_{4.5}$ 1.12 mmol/l; total ammonia 0.01 mg/l; nitrates NO_3^- 5.11 mg/l; nitrites NO_2^- 0.006 mg/l; phosphate PO_4^{3-} 0.03mg/l; chemical oxygen demand COD_{Mn} 0.84 mg/l; oxygen saturation 95–99%; pH 7.7–7.9.

Haematological profile

At the end of the treatment, blood was drawn from the *vena caudalis* using a heparinised syringe (Heparin inj.; Léčiva, Prague, Czech Republic) and immediately after sampling was analysed to determine the erythrocyte count (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular

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volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and leukocyte count (Leuco).

Biochemical blood plasma profile

Blood was separated by centrifugation at 1 073 × *g* for 10 min at 4 °C. The plasma samples were kept at –80 °C 14 days pending analysis. The biochemical blood profile was determined using a Vettest 8008 biochemical analyser (IDEXX Laboratories Inc., Westbrook, USA). The evaluated indices included the glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB), ammonia (NH_3), triglyceride (TAG), aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate (LACT), lactate dehydrogenase (LDH), calcium (Ca^{2+}), magnesium (Mg), and phosphorus (PHOS).

Oxidative stress and antioxidant biomarkers

After the blood sampling, the fish were killed by severing the spinal cord, and gill, liver, and muscle samples were taken to evaluate the oxidative stress and antioxidant enzymes. The tissues were quickly removed, immediately frozen, and stored at –80 °C for 28 days pending analysis. Before analysis, the frozen tissue samples were weighed and homogenised (1 : 10 w/v) (Ultra Turrax; Ika, Staufen, Germany) in 50 mM of a cooled potassium phosphate buffer, pH 7.0, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). Ice was used for cooling during the homogenisation. The homogenate was halved, with a portion for thiobarbituric acid reactive substances (TBARS) and another centrifuged at 12 000 × *g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for the antioxidant enzyme analyses.

The oxidative damage was evaluated by lipid peroxidation, calculated from the TBARS assay according to Lushchak et al. (2005). The total superoxide dismutase activity (SOD) was determined spectrophotometrically at 420 nm (Marklund and Marklund 1974). The catalase activity (CAT) was measured spectrophotometrically at 240 nm according to Beers and Sizer (1952). The glutathione reductase activity (GR) was determined spectrophotometrically by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Carlberg and Mannervik 1975). The glutathione peroxidase (GPx) activity was assayed from the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Lawrence and Burk 1976). The protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as the standard.

Histology

After the blood collection, the gill samples were removed and immediately fixed in 10% formalin prepared with standard histological techniques, stained with haematoxylin and eosin, and examined by light microscopy. Histological alterations were scored as (–) no pathology; (+) pathology in < 20% of the fields; (++) pathology in 20–60% of the fields, and (+++) pathology in > 60% of the fields.

Statistical analysis

Prior to the analysis, all the data were checked for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If the conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to evaluate the differences in the measured variables among the experimental and control groups. When a difference was detected ($P < 0.05$), Tukey's unequal N-HSD test was applied. If the conditions for the ANOVA were not satisfied, the non-parametric Kruskal-Wallis test was used.

RESULTS

Haematological profiles

The carp exposed to FBZ, LHC or IVM did not differ from the controls with respect to the haematological profile (Table 1).

Plasma biochemical profile

A two-hour LHC bath was associated with significantly higher plasma GLU, LACT and NH_3

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Table 1. Haematological profiles of the common carp (n = 6) exposed to the FBZ (25 mg/l, 12 h), LHC (50 mg/l, 2 h) or IVM (0.031 mg/l, 12 h) therapeutic baths

Parameter	C	LHC	FBZ	IVM
RBC (T/l)	1.4 ± 0.1	1.3 ± 0.4	1.5 ± 0.1	1.5 ± 0.1
Hb (g/l)	65.4 ± 9.4	65.0 ± 7.9	67.3 ± 8.5	68.5 ± 6.7
PCV (l/l)	0.3 ± 0.04	0.3 ± 0.05	0.3 ± 0.04	0.3 ± 0.03
MCV (fl)	235.0 ± 27.3	215.9 ± 57.3	207.1 ± 27.9	200.1 ± 25.9
MCH (pg)	47.7 ± 8.5	53.5 ± 17.3	43.8 ± 5.1	45.1 ± 4.6
MCHC (g/l)	204.0 ± 33.8	251.0 ± 70.6	214.1 ± 32.8	228.6 ± 35.7
Leuco (G/l)	55.6 ± 19.2	41.1 ± 23.3	51.0 ± 17.4	40.3 ± 8.1

Data are mean ± SD, *Indicates difference from the control at $P < 0.05$; **Indicates difference from the control at $P < 0.01$
C = control; FBZ = fenbendazole; Hb = haemoglobin; IVM = ivermectin; Leuco = leukocyte count; LHC = levamisole; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; PCV = packed cell volume; RBC = erythrocyte count

($P < 0.01$), ALP and ALT ($P < 0.05$), and a significantly lower AST activity ($P < 0.01$) compared to the control. The levels of the TP, ALB, GLOB, TAG, LDH, CK, Ca^{2+}, Mg and PHOS in the LHC-treated fish did not differ from that of the controls (Table 2). No differences, from the control, were observed in the carp plasma biochemical profile with the FBZ or IVM treatment.

Oxidative stress and antioxidant biomarkers

The LPO levels, calculated from the TBARS, in the muscles and liver of the carp after the 2-h exposure to the LHC were significantly higher ($P < 0.01$) than in the control group. The SOD activity in the muscles ($P < 0.01$), and the CAT activity in the liver ($P < 0.05$) were significantly higher, and

Table 2. Plasma biochemical profile of the common carp (n = 6) exposed to the FBZ (25 mg/l, 12 h), LHC (50 mg/l, 2 h) or IVM (0.031 mg/l, 12 h) therapeutic baths

Parameter	C	LHC	FBZ	IVM
GLU (mmol/l)	5.0 ± 0.6	7.2 ± 1.2**	5.1 ± 1.0	4.8 ± 0.8
TP (g/l)	36.2 ± 3.3	37.0 ± 3.3	36.3 ± 2.5	35.2 ± 3.6
ALB (g/l)	2.2 ± 0.7	1.8 ± 1.2	2.0 ± 0.8	2.2 ± 0.7
GLOB (g/l)	34.0 ± 2.8	35.2 ± 3.9	34.3 ± 2.8	33.0 ± 3.5
NH_3 (µmol/l)	400.0 ± 52.6	660.8 ± 77.6**	534.7 ± 140.4	382.8 ± 68.7
TAG (mmol/l)	0.9 ± 0.2	0.8 ± 0.2	1.0 ± 0.2	0.7 ± 0.2
AST (µkat/l)	1.3 ± 0.5	0.8 ± 0.4*	1.2 ± 0.2	1.3 ± 0.6
ALT (µkat/l)	0.1 ± 0.02	0.2 ± 0.05*	0.10 ± 0.08	0.1 ± 0.03
LDH (µkat/l)	16.4 ± 3.2	18.9 ± 2.9	13.3 ± 4.5	14.7 ± 3.1
CK (µkat/l)	15.8 ± 4.5	14.4 ± 3.7	16.9 ± 1.8	15.5 ± 2.9
LACT (mmol/l)	0.8 ± 0.4	3.1 ± 2.1**	1.5 ± 0.8	1.7 ± 0.9
ALP (µkat/l)	0.1 ± 0.05	0.3 ± 0.10**	0.2 ± 0.04	0.1 ± 0.07
Ca^{2+} (mmol/l)	2.5 ± 0.3	2.8 ± 0.5	2.6 ± 0.2	2.6 ± 0.4
Mg (mmol/l)	1.3 ± 0.6	1.4 ± 0.6	1.5 ± 0.3	1.3 ± 0.3
PHOS (mmol/l)	1.7 ± 0.4	1.5 ± 0.3	1.5 ± 0.4	1.8 ± 0.5

Data are presented as mean ± SD; *Indicates difference from the control at $P < 0.05$; **Indicates difference from the control at $P < 0.01$
ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate transaminase; C = control; Ca^{2+} = calcium; FBZ = fenbendazole; GLOB = globulin; GLU = glucose; IVM = ivermectin; LACT = lactate; LDH = lactate dehydrogenase; LHC = levamisole; Mg = magnesium; NH_3 = ammonia; PHOS = phosphorus; TAG = triglyceride; TP = total protein

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Table 3. Oxidative stress and antioxidant indices in tissue of the common carp ($n = 6$) exposed to the FBZ (25 mg/l, 12 h), LHC (50 mg/l, 2 h) or IVM (0.031 mg/l, 12 h) therapeutic baths

Parameter	Tissue	C	LHC	FBZ	IVM
TBARS (nmol/mg protein)	gill	0.4 ± 0.06	0.4 ± 0.09	0.4 ± 0.09	0.4 ± 0.03
	liver	0.4 ± 0.06	0.6 ± 0.12**	0.5 ± 0.12	0.5 ± 0.08
	muscle	0.8 ± 0.24	1.5 ± 0.07**	0.9 ± 0.16	0.9 ± 0.19
SOD (nmol NBT/min/mg protein)	gill	0.4 ± 0.09	0.2 ± 0.03	0.4 ± 0.24	0.3 ± 0.20
	liver	0.1 ± 0.1	0.2 ± 0.04	0.2 ± 0.05	0.2 ± 0.12
	muscle	0.05 ± 0.03	0.1 ± 0.04**	0.03 ± 0.02	0.1 ± 0.03**
CAT (μmol H_2O_2/min/mg protein)	gill	0.5 ± 0.07	0.6 ± 0.13	0.6 ± 0.09	0.6 ± 0.09
	liver	2.0 ± 0.49	3.1 ± 0.85*	1.9 ± 0.43	2.1 ± 0.68
	muscle	0.1 ± 0.05	0.2 ± 0.05	0.2 ± 0.04	0.2 ± 0.06
GR (nmol NADPH/min/mg protein)	gill	0.5 ± 0.26	0.2 ± 0.03**	0.3 ± 0.08	0.4 ± 0.16
	liver	0.2 ± 0.06	0.05 ± 0.01**	0.1 ± 0.02	0.1 ± 0.045
	muscle	0.2 ± 0.03	0.004 ± 0.003*	0.1 ± 0.07	0.1 ± 0.08
GPx (nmol/mg protein)	gill	0.6 ± 0.24	0.6 ± 0.13	0.5 ± 0.15	0.5 ± 0.19
	liver	1.2 ± 0.16	1.1 ± 0.15	1.2 ± 0.37	1.1 ± 0.16
	muscle	0.3 ± 0.07	0.3 ± 0.07	0.3 ± 0.08	0.3 ± 0.20

Data are presented as mean ± SD; *Indicates difference from control at $P < 0.05$; **Indicates difference from control at $P < 0.01$

C = control; CAT = catalase activity; FBZ = fenbendazole; GR = glutathione reductase activity; IVM = ivermectin; LHC = levamisole; NADPH = nicotinamide adenine dinucleotide phosphate; NBT = nitro blue tetrazolium; PGx = glutathione peroxidase; SOD = superoxide dismutase activity; TBARS = thiobarbituric acid reactive substance

the GR activity in the gills ($P < 0.01$), liver ($P < 0.01$), and muscles ($P < 0.05$) were significantly lower than the control (Table 3).

The carp exposed to FBZ did not exhibit differences from the control in the oxidative stress and antioxidant indices of the gills, liver, or muscles.

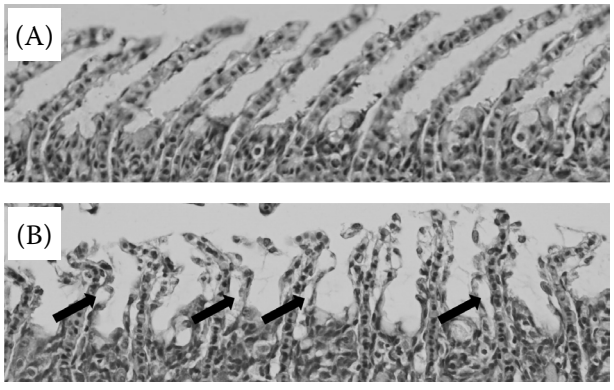

Figure 1. Gills of the carp (*Cyprinus carpio* L.) after ivermectin exposure

Haematoxylin and eosin stain, × 400. (A) Control group. (B) IVM group (0.031 mg/l, 12 h). The arrows indicate lifting of epithelial cells

The carp exposed to IVM showed a significantly higher total SOD activity in the muscles compared to the controls ($P < 0.01$).

The TBARS, CAT, GR, and GPx levels in all the tissues were comparable to the controls.

Histology

The examination of the gills revealed a slight pathology (++) in groups exposed to IVM compared to the control, including focal epithelial lifting and hyperaemia of the lamellar vessels (see Figure 1). No histological differences from the controls were demonstrated with the other treatments.

DISCUSSION

The haematological profile can provide invaluable information on the physiological and pathological changes in fish (Masopust 2000). We observed no significant differences from the controls in the

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haematological profile of the common carp after exposure to FBZ, LHC or IVM. Levamisole has been widely shown to have immunostimulatory effects on farmed fish, including enhancing the circulating leukocytes and phagocytic activity, indicating mild erythropenia, intralienic haemolysis, and worsening of the health status (Wijendra and Pathiratne 2011). Oliveira et al. (2019a) observed a decrease in the red blood cell (RBC) and thrombocyte counts in dog snapper *Lutjanus jocu* after an oral LHC treatment at 5 mg/kg fish body weight. Levamisole was reported to influence the haematological parameters including the haemoglobin, white blood count, and differential leukocyte count in juvenile pacu *Piaractus mesopotamicus* (Sado et al. 2010). Oliveira et al. (2019b) observed a decrease in the PCV, Hb, MCV and MCHC in *C. macropomum* after an IVM treatment. An ivermectin treatment was shown to decrease the RBC, PCV, and Hb in the North African catfish *Clarias gariepinus* (Ogueji et al. 2019).

The therapeutic LHC bath was associated with an increase in the GLU, LACT, NH_3, ALP, ALT and a decrease in the AST activity in the carp blood plasma. The increase in the blood GLU and LACT concentrations demonstrates a response to metabolic stress (Wendelaar Bonga 1997). Our findings agree with those of Pahor-Filho et al. (2017), Oliveira et al. (2019a), Oliveira et al. (2019b), and Sadati et al. (2021) who also detected an increase in the glucose or lactate concentration in Serrasalmidae (pacu, dog snapper) and beluga (*Huso huso)* following an LHC treatment. In contrast, an LHC treatment was reported not to affect the glucose concentration in tambaqui (da Silva et al. 2021) and juvenile pacu (Sado et al. 2010). An increased ammonia concentration after an LHC treatment indicates an inability of the organism to convert the toxic ammonia to less harmful substances. Differences in the transaminase activity in LHC-treated carp reflect stress during the bath treatment.

The therapeutic baths in FBZ and IVM did not affect the carp plasma biochemical profile. However, in contrast, the IVM treatment at 9, 13, 17, 21, and 25 µg/l was found to increase the ALT, AST, and ALP activity in North African catfish (Ogueji et al. 2020), which suggests stress and liver cell damage to the fish following the IVM and FBZ treatment.

We only observed that the LHC treatment lead to oxidative stress provoking a significant increase in the LPO in the muscles and liver of the carp. Our results indicate that the reactive oxygen species (ROS) may be associated with the metabolism of the LHC and consequent increased production of ROS to the point that the antioxidant enzymes could not counteract the peroxidation of the carp membrane lipids. Ogueji et al. (2020) reported an increase in the LPO in North African catfish after an IVM treatment.

Our results showed antioxidant biomarkers in the carp tissues as a consequence of the LHC and IVM treatments. A twelve-hour exposure to 25 mg/l FBZ did not affect the antioxidant indices in the carp gills, liver, or muscles. Induction of antioxidant biomarkers in carp tissues after an LHC and IVM treatment could be an adaptive response to the stress that neutralises the impact of the generated ROS. A similar observation was reported by Li et al. (2006), Sakin et al. (2012), Ogueji et al. (2020), and Gupta et al. (2020).

During the histological examination, we observed mild focal epithelial lifting and hyperaemia of the lamellar vessels in gills exposed to IVM. The changes in the gill morphology after the IVM bath were slight, and all the tested antiparasitic baths can be considered safe for carp with respect to the gill tissue damage.

In conclusion, it can be stated that the LHC therapeutic bath, under the test conditions, affects the haematological profile, plasma biochemistry, and antioxidant indicators and causes oxidative stress in the carp.

Ivermectin, in the bath, does not affect the haematological and biochemical blood profile, however, it causes changes in the SOD and the histology of the carp gills. The FBZ bath, under the test conditions, does not affect the carp physiology and can be recommended for treatment. However, the final choice of veterinary drugs must consider the effectiveness, legislation, availability, cost, ease of use, and safety for the user and the environment. Levamisole, fenbendazole, and ivermectin are not approved for use in fish for human consumption, but MRLs have been established for the meat of sheep, goats, pigs, cattle, and poultry.

Therefore, it is possible to use them based on the off-label principle as a VMP intended for other food animals.

When such off-label use is considered, a tolerance test must be conducted before administration and the specified withdrawal period of at least 500 degree-days must adhered to.

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Conflict of interest

The authors declare no conflict of interest.

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