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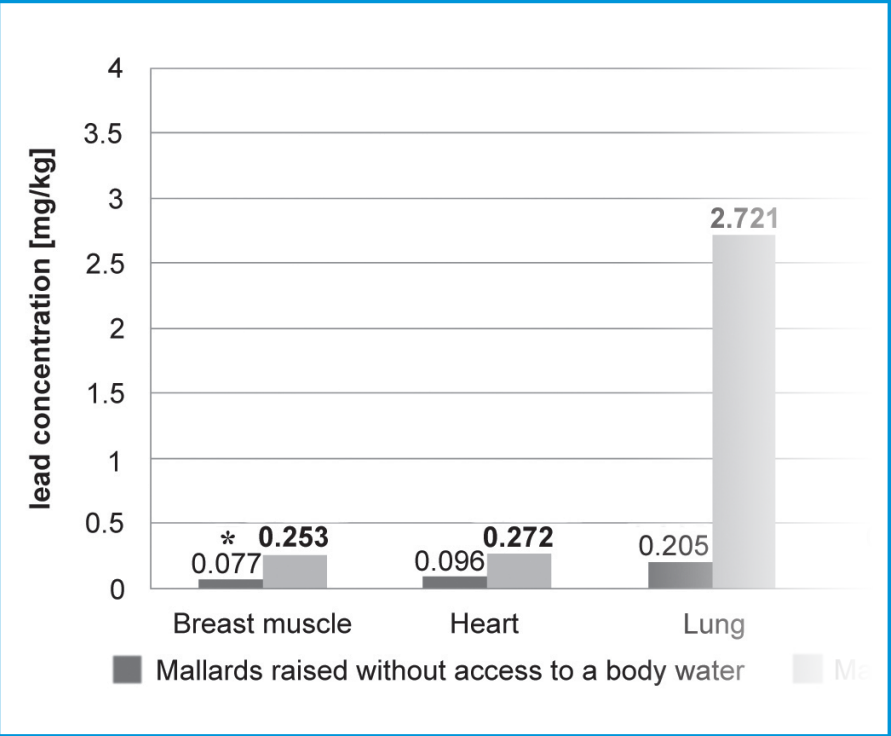
SLOVENSKI VETERINARSKI ZBORNIK



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Review Article

THE EFFECT OF SELECTED TRIAZINES ON FISH: A REVIEW

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Summary: Anthropogenic pollution constitutes a worldwide problem of growing concern. Increased environmental pollution can be attributed to a variety of factors associated with industrial and agricultural technologies. Triazine herbicides are among the most commonly used pesticides in the world, and are predominant class of herbicide. In recent years, concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection these herbicides compounds and their of residual concentrations in different environmental compartments. The detectable levels are in drinking and ground water, food and fish, also their metabolites are frequently found in water ecosystems. Moreover, some of triazine pesticides are prohibited in European country. Eight s-triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and they are included in the European Union Priority Pollutants List and the U.S. Environmental Protection Agency's List. Current knowledge about residual triazine in the aquatic environment, including status, toxic effects, and traizne in fish, are reviewed. Based on the above, we identify major gaps in the current knowledge and some directions for future research. A review contains the impact of the seven most frequently detected triazines in water (ametryne, atrazine, metribuzine, prometryne, simazine, terbuthylazine, and terburyne) on fish physiology and acute toxicity. Toxic effect of triazine has influence mainly on growth, early development, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney of fish.

Key words: triazine; fish; toxicity; biochemical profile; hematology; histology

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Abbreviations & Units: AChE – acetylcholinesterase; ACP – acyl carrier protein; ALB – albumin; ALP – alkaline phosphatase; ALT – alanine aminotransferase; APND – aminopyrine; AST – aspartate aminotransferase; Ca – calcium; CA – carbonic anhydrase; CAT – catalase; CbE – carboxylesterase; CF – condition factor; CK – creatine kinase; CREA – creatine; CYP – cytochrome; DS – distal segments; EC – ceruloplasmin; ERND – erythromycin N-demethylase; EROD – ethoxyresorufin-O-deethylase; FRAP – ferric reducing ability of plasma; GLOB – total globulins; GLU – glucose; GSH – reduced glutathione; GPx – glutathione peroxidase; GR – glutathione reductase; Hb – hemoglobin; MRCs – mitochondria-rich cells; HSI – hepatosomatic index; Hsp – heat shock protein; iNOS – inducible nitric oxide synthase; LACT – lactate; LC50 – lethal concentration; LDH – lactate dehydrogenase; LPO – lipid peroxide; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; MCV – mean corpuscular volume; MDA – malondialdehyde; Mg – magnesium; Na – natrium; NCR – NADPH cytochrome P450 reductase; NH3 – ammonia; P – phosphorus; PCV – hematocrit; PD – proximal segments; PHOS – inorganic phosphate; POD – guaiacol peroxidase; PROD – pentoxyresorufin-O-deethylase; RBC – erythrocyte count; RCs – rodlet cells; ROS – reactive oxygen species; SOD – superoxide dismutase; SSI – spleen somatic index; SW – spleen weight; TAG – triacylglycerols; TBARS – thiobarbituric acid reactive substances; TP – total protein; UDPGT – UDP-glucuronosyltransferase; WBC – leukocyte count; 11-KT – 11-ketotestosterone.

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Introduction

Sources of pollution constitute a problem of increasing concern all over the world (1). Increased environmental pollution can be attributed to a variety of factors resulting from different industrial and agricultural technologies (2). Agricultural development has led a parallel growth in the use of chemical agents for plague controls, which are known as pesticides. These compounds are released into the environment and due to their physico-chemical properties, such as water solubility, vapor pressure or partition coefficients between organic matter (soil or sediment) and water, they can disperse in various environmental media provoking serious health problems (3).

Effects of the residues of various substances persisting in the aquatic environment, the most important of those being pesticides, also are monitored. From among pesticides, the most frequently found are residue of triazine herbicides. Triazine herbicides are among the most commonly used pesticides in the world. The triazine was discovered in 1954 (4). The chemical structure of triazines is divided into asymmetric (metribuzine) and symmetric (atrazine, simazine, prometryne, etc.). The structures of all of the triazine herbicides have a six-member ring containing three nitrogen atoms and three carbon atoms (5). Triazines compounds are used against a wide variety of weed species. They are used primarily to selective control broad leaf and grassy weeds (6). As herbicides, the triazines may be used alone or in combination with other herbicide active ingredients to increase the weed control spectrum (7).

In recent years, concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in groundwater and in different environmental compartments (8, 9). Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community (10) and they are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. Triazine are highly toxic to moderately toxic to fish (Tab. 1.). On base of these informations, we decided to write a review about the impact of the seven most frequently detected triazines in water (ametryne, atrazine, metribuzine, prometryne, simazine, terbuthylazine, and terburyne) on fish.

Ametryne

Ametryne (4-N-ethyl-6-methylsulfanyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine) was first registered as a pesticide use to control broadleaf weeds and annual grasses in sugarcane fields in the USA in 1964. Ametryne has also been used as a general herbicide in uncultivated areas, rights of way, and industrial areas and aquatic weeds. Over time, the uses of ametryne have been cancelled so that only four use sites remain: field corn, popcorn, pineapple, and sugarcane. Currently, only one ametryne end use product is registered. In 2005 US EPA has received requests for voluntary cancellation of all other products (37). The extensive use of ametryne in agriculture and some properties of this herbicide such as aerobic soil halflife of 53.2 days, adsorption coefficient of 3.45, and leaching potential of 6.94 (38) suggest that it could be present in the environment as a potential contaminant of soil, surface water and groundwater, and river sediment (39).

Environmental fate

Ametryne is a moderately persistent herbicide which inhibits photosynthesis and other enzymatic processes. The environmental fate of ametryne varies based on the site-specific properties of the soil to which it is applied. Based on packed soil column leaching studies, ametryne and its degradates exhibit moderate to high mobility in most sandy to loamy soils, except for clay where its mobility is low. The major route of degradation of ametryne is aerobic soil metabolism, with an observed half-life range of 9.6 days to 84 days. Ametryne is stable to hydrolysis, and degrades slowly by aquatic photolysis, half-life is 368 days (37). Major metabolite product of ametryne is deethyl ametryne (38).

Ametryne is persistent, it may leach as a result of high rainfall, floods, and furrow irrigation. Given its persistence and mobility, transport of ametryne to ground water and surface water is expected. Monitoring of ametryne concentrations in ground water and surfase water is limited. In Europe rivers ametryne levels can reach values,

Table 1: Acute toxicity of triazines on fish

Exposure 96hLC50 [mg/L] (Reference)							
Species	**Ametryne**	**Atrazine**	**Metribuzine**	**Prometryne**	**Simazine**	**Terbuthylazine**	**Terbutryne**
Guppy (*Poecilia reticulata*)	0.3 (11)	4.3 (13)	-	7.0*** (29)	-	1.6 (13)	-
Japanese eel *(Anguilla japonica*)	1.5** (12)	-	-	-	-	-	-
Rainbow trout (*Oncorhynchus mykiss*)	3.4 (13)	8.8 (13)	42.0 (24)	2.9 (14)	100.0* (14)	3.4 (14)	3.0 (13)
Sheepshead minnow (*Cyprinodon variegatus*)	5.8 (14)	13.4 (14)	85.0 (14)	5.1 (28)	4.3 (14)	-	-
Goldfish (*Carassius auratus*)	14.0 (14)	58.6 (22)	-	4.0 (14)	32.0 (14)	-	-
Fathead minnow (*Pimephales promelas*)	16.0 (14)	4.1 (15)	-	-	-	-	-
Bluegill (*Lepomis macrochirus*)	19.0 (13)	50.0 (13)	76.0 (14)	7.9 (28)	100.0 (34)	7.5 (14)	4.0 (13)
Black bullhead (*Ameiurus melas*)	25.0 (11)	35.0 (11)	-	3.0 (11)	65.0 (11)	7.0 (11)	3.0 (11)
Crucian carp (*Carassius carassius*)	27.0 (11)	100.0** (11)	-	-	100.0 (13)	66.0 (13)	4.0 (11)
Channel catfish (*Ictalurus punctatus*)	-	10.0 (16)	3.4 (23) 100.0 (24)	-	85.0 (14)	-	-
Coho salmon (*Oncorhynchus kisutch*)	-	12.0 (17)	-	-	-	-	-
Common carp (*Cyprinus carpio*)	-	18.8 (18)	175.1 (26)	8.0 (27)	40.0** (33)	-	4.0 (35)
Chinook salmon (*Oncorhynchus tshawytscha*)	-	19.0 (17)	-	-	910.0 (17)	-	-
Fera (*Coregonus fera*)	-	26.3 (19)	-	-	-	-	-
Brown trout (*Salmo trutta*)	-	27.0 (20)	-	-	70.0 (20)	-	-
Zebrafish (*Danio rerio*)	-	40.0** (21)	-	3.0 (27)	12.6 (31)	-	-
Red rasbora (*Rasbora heteromorpha*)	-	-	140.0 (25)	-	-	-	-
Red-tailed rasbora (*Rasbora borapetensis*)	-	-	145.0 (25)	-	-	-	-
Minnow (*Phoxinus phoxinus*)	-	-	-	4.5 (27)	-	-	-
Silver carp (*Hypophthalmichthys molitrix*)	-	-	-	7.0 (27)	-	-	-
Western mosquitofish (*Gambusia affinis*)	-	-	-	10.0* (30)	-	-	-
Tilapia mosambicus (*Oreochromis mossambicus*)	-	-	-	-	3.1 (31)	-	-
Barbus ticto (*Barbus ticto*)	-	-	-	-	24.5 (31)	-	-
Rohu (*Labeo rohita*)	-	-	-	-	26.9** (32)	-	-
Yellow bullhead (*Ameiurus natalis*)	-	-	-	-	110.0 (14)	-	-
genus Bullheads (*Ameiurus sp.*)	-	-	-	-	-	7.0 (13)	-
Perch (*Perca fluviatilis*)	-	-	-	-	-	-	4.0 (11)
Grass carp (*Ctenopharyngodon idella*)	-	-	-	-	-	-	8.9** (36)

* 24hLC50; ** 48hLC50; *** 72hLC50

up to 1.14 μg/L (39-41). In surface water near to Sao Paulo (Brasil) was found contamination from 0.17 to 0.23 μg/L (42, 43).

Acute toxicity

Ametryne is highly toxic to moderately toxic to fish. The lethal concentration (96hLC50) for fish is in range 0.3 to 27.0 mg/L (Tab. 1.). Ametryne is highly toxic to crustaceans and moderately to highly toxic to mollusks (44).

Effect of ametryne on fish

Although the lethal toxicity of fish to ametryne, have been well-documented, there is a dearth of data on the effects of ametryne on fish physiology. Only three studies on effects on fish physiology of ametryne have been conducted. Ametryne caused increase of plasma glucose level, hepatic glucose-6-phosphatase and decreased of muscle and liver glycogen contents in grass carp (*Ctenopharyngodon idella*) during sublethal and lethal (96hLC50) exposure (45). Acute exposure of ametryne inhibited of cholinesterase in juvenile and adult zebrafish (*Danio rerio*). Ametryne caused increase of acivity glutathione S-transferase only in larvae, but not in adult fish. And they conclude that these biomarkers are a useful tool to evaluate the risk of fish exposure of ametryne, even at sublethal levels (46). Mix atrazine and ametryne in concentrations (0.5, 1.0, 1.5, and 2.0 μg/L) exposure caused micronuclei formation and erythrocytic nuclear abnormalities in zebrafish (47).

Atrazine

Atrazine (6-chloro-N2-ethyl-N4-(1-methylethyl)-1,3,5-triazine-2,4-diamine) was used for control of some annual broadleaf and grass weeds in corn, sorghum, sugar cane, orchards, vineyards and non-agricultural areas (48). Atrazine causes blockage of electron transport by Hill´s reaction in plant photosynthesis (49). It is an indirect endocrine disruptor (50, 51) because it can cause convert testosterone to estrogen (52). Atrazine and plant protection products containing this substance were banned in 2005 by Commission Decision 2004/247/CE.

Environmental fate

Atrazine is toxic, persistent and bioaccumulative (53). According to its physical and chemical characteristics of the group of compounds that are moderately resistant and moderately mobile in soils. The half-life of atrazine, depending upon the environment and the amount and frequency of administration, varies between a few days to several months. The photolysis in water is very slow. An estimated half-life is 805 days. In controlled aerobic water-sediment systems atrazine was eliminated from the water with a half-life of 28-134 days, while the degradation half-life was found to be 45-253 days for the whole system (54). In European rivers atrazine levels can reach values, up to 6.47 μg/L (55), but in US rivers was about 20 μg/L (56).

Acute toxicity

Lethal acute toxicity (96hLC50) of atrazine for fish is ranging from units to hundreds milligrams per liter (Tab. 1.). Order of sensitivity to atrazine is: macrophytes > phytoplankton > zooplankton > fish > benthos (57). Fish subjected to acute exposure of atrazine herbicide displayed uncoordinated behavior. At the initial exposure, fish were alert, stopped swimming and remained static in position in response to the sudden changes in the surrounding environment. After some time they tried to avoid the toxic water with fast swimming and jumping. Faster opercula activity was observed as surfacing and gulping for air. They secreted copious amounts of mucus from whole body continuously and soon a thick layer of mucus was found deposited in the buccal cavity and gills. Body pigmentation was decreased. Ultimately fish lost their balance, consciousness, engage in rolling movement and became exhausted and lethargic. Lastly, they remained in vertical position for a few minutes with anterior side or terminal mouth up near the surface of the water, trying to gulp air and tail in a downward direction. Soon they settled at the bottom of the tank, and after some time their bellies turned upward and the fish died (58).

Table 2: The effect of atrazine on common carp

Development stage	Concentration	Exposure	Effects	Reference
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ EROD, PROD, CYP, CYP1A mRNA level in liver	(61)
Juvenile	5 mg/L	96 hours	↑ GLU; ↓ RBC, WBC	(62)
	15 mg/L		↑ GLU, TP, ALB, ALT, ALP, LDH, myclocytes ↓ WBC, lymfocytes	
	20 mg/L		↑ GLU, TP, ALT, ALP, LDH, myclocytes, ↓ P, Ca, WBC, lymfocytes	
	30 mg/L		↑ GLU, ALT, AST, LDH, myclocytes, monocytes; injection of visceral vessels, ↓ PCV, RBC, Hb, WBC, lymfocytes; dystrophic lesions of hepatocytes, teleangiectasis in gill	
Juvenile	4.28 µg/L	40 days	↑ ACP in spleen, ACP in head kidney ↓ Na+/K+-ATPase in head kidney	(63)
	42.8 µg/L		↑ ACP in spleen, ACP in head kidney ,MDA in spleen, ↓ SOD in spleen, SOD in spleen, head kidney, Na+/K+-ATPase in head kidney	
	428 µg/L		↑ ACP in spleen, ACP in head kidney, MDA in spleen, head kidney ↓ ALP in spleen, ALP in head kidney, Na+/K+-ATPase in spleen, Na+/K+-ATPase in head kidney, SOD in spleen, SOD in head kidney	
Juvenile	4.28 µg/L	40 days	↑ HSP90	(64)
	4.28, 42.8, 428 µg/L	40, 80 days	↑ HSP60	
	42.8, 428 µg/L		↑ HSP70	
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ APND, ERND, mRNA levels of CYP1 family (CYP1A, CYP1B, CYP1C) in gill	(65)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↑ iNOS, production of NO in brain	(66)
Juvenile	428 µg/L	40 days	↓ AChE, mRNA levels of AChE	(67)
Juvenile	42.8, 428 µg/L	40 days	↑ MDA in kidney, MDA in brain; ↓ CAT in kidney, SOD in kidney, SOD in brain, GSH-Px in kidney; GSH-Px in brain; different degrees of granule cell loss in the hippocampus, reduction of Nissl bodies, degeneration of Purkinje cells, neuropil loss; swelling of epithelial cells of renal tubules, necrosis in the tubular epithelium, contraction of the glomerulus and expansion of Bowman's space,	(68)
Juvenile	4.28 µg/L	40 days	↑ CAT in gill; CAT in liver ↓ GSH-Px in liver	(69)
	42.8, 428 µg/L		↑ MDA in liver, MDA in gill ↓ CAT in liver, CAT in gill; SOD in liver, SOD in gill, GSH-Px in liver, GSH-Px in gill; different degrees of hydropic degeneration of liver , vacuolisation, pyknotic nuclei, and fatty infiltration; varied degrees of epithelial hypertrophy in gill, telangiectasis, oedema with epithelial separation from basement membranes, general necrosis, and epithelial desquamation	
Juvenile	428 µg/L	40 days	↑ mRNA levels of IL-1 beta, mRNA levels of IL-1R1	(70)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↓ RNA levels of AChE in brain and muscle	(71)
Juvenile	4.28, 42.8, 428 µg/L	40 days	↓ AChE, CbE in brain and muscle	(72)
Juvenile	< 7 µg/L	14 days	induction cytochrome P4501A1	(73)
	< 100 µg/L		↑ DNA strand breaks	
Embryo - larvae	0.3 µg/L	30 days	↑ GPx, GST, SOD, CAT, GR	(74)
	30 µg/L		↓ GR	
	100, 300 µg/L		↑ TBARS, ↓ GR	

Table 3: The effect of atrazine on zebrafish

Development stage	Concentration	Exposure	Effects	Reference
Juvenile	0.3 µg/L	28 days	↑ GPx, GR; ↓ CAT	(75)
	3 µg/L		↑ GPx; ↓ CAT	
	30 µg/L		↑ GPx, GR, SOD, TBARS; ↓ CAT	
	90 µg/L		↑ GPx, SOD, TBARS; ↓ CAT	
	25 µg/L		scattered lesions in gill	
Juvenile	90 µg/L	28 days	↓ growth rates; dystrophic lesions of hepatocytes; ↑ MRCs in filament epithelium of gill	(76)
Juvenile	2.5 µg/L	21 days	↑ SOD, CAT	(77)
	2.5, 5, 10 µg/L	14, 21 days	↑ POD	
Adult – female	10 µg/L	14 days	↑ SOD in ovary, CAT in ovary; ↓ GSH in liver	(78)
	100 µg/L		↑ SOD in liver, MDA in liver; ↓ GSH in liver	
	1000 µg/L		↑ SOD in liver, CAT in liver, MDA in liver; ↓ GSH in liver	
Adult – female	0.01, 0.1, 1 mg/L	10, 15 days	↑ cytochrome P450 content, APND, ERND	(79)
	0.01, 0.1, 1 mg/L	20, 25 days	↑ APND, ERND, NCR	
Adult – male	0.01, 0.1, 1 mg/L	10, 15 days	↑ cytochrome P450 content, NCR, APND, ERND	
	0.1 mg/L	20, 25 days	↑ cytochrome P450 content, APND	
Embryo - larvae	4 mg/L	48 hours	disturbed the normal development to long pec stage	(80)
	10-20 mg/L		retardations in organogenesis, a slowdown of movements, and functional disturbances of heart and circulatory system	
Embryo - larvae	5 mg/L	48 hours	↑ soluble (s) and microsomal (m) GST	(81)

Effect of atrazine on fish

Effects of atrazine on fish physiology, have been well-documented. Its effect is the best described from all triazines. Atrazine affected hematological, biochemical profile, antioxidant enzymes, oxidative stress indices, growth and caused histopatological changes in tissues. The effects of atrazine are mentioned on carp (Tab. 2.), zebrafish (Tab. 3.), Salmonidae (Tab. 4.), other fish (Tab. 5.). In a study conducted by Ventura et al. (59), it was observed that the herbicide atrazine has a genotoxic and mutagenic effect. In this study, the authors observed that the herbicide can interfere in the genetic material of the organisms exposed, even at doses considered residual, which led the authors to suggest that residual doses of atrazine, resulting from leaching of soils of crops near water bodies, can interfere in a negative form in the stability of aquatic ecosystems. The bioaccumulation factors for atrazine in the liver, muscle, heart, gonads and brain of banded tilapia (*Tilapia sparrmanii*) is ranged from 0.9 to 20.0 (60).

Metribuzine

Metribuzine (4-amino-6-tert-butyl-3-(methythio)-1,2,4-triazin-5-one) is an asymmetrical triazine herbicide. It is distinct from the symmetrical

Table 4: The effect of atrazine on Salmonidae

Species	Concentration	Exposure	Effects	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	555 µg/L	4 days	↑ cortisol, monocytes; ↓ SSI, lymphocytes	(82)
Atlantic salmon (*Salmo salar* L) Smolts	100 µg/L	21 days	↓ feeding, Cl^-, Mg^{2+}, Na^+, Ca^{2+}; ↑ cortisol	(83)
Atlantic salmon (*Salmo salar* L) Smolts	2 µg/L	7 days	↓ Na^+K^+ATPase in gill	(84)
	5, 10 µg/L		↑ cortisol; ↓ Na^+K^+ATPase in gill	
Atlantic salmon (*Salmo salar* L) Smolts	atrazine (1 µg/L) + 4-nonylphenol (5 µg/L)	7 days	↑ Na^+K^+ATPase in gill, plasma Cl^-, Na^+	(85)
	atrazine (2 µg/L) + 4-nonylphenol (10 µg/L)		↑ plasma Cl^-, Na^+; ↓ Na^+K^+ATPase in gill	
Atlantic salmon (*Salmo salar* L.) Adult - male	above 0.04 µg/L	shorten	↓ 17,20 beta-dihydroxy-4-pregnen-3-one in plasma and milt	(86)
Rainbow trout (*Oncorhynchus mykiss*) Renal tubules	10, 20, 40, 80, 160 µg/L	4 weeks	In PS I - proliferation of smooth endoplasmic reticulum, atypical mitochondria and lysosomes, as well as gradual alterations of the apical plasmalemma; In PS II - cells proliferation of peroxisomes, ring- and cup-shaped mitochondria, alterations in the basal labyrinth; in DS cells, proliferation of atypical mitochondria with longitudinally oriented cristae, disorganization of Golgi fields and vacuolization of the cell base.	(87)

triazines such as atrazine and simazine, in which the central ring structure has alternating carbon and nitrogen atoms, in that metribuzin possesses two nitrogen atoms and two adjacent carbon atoms. It was first registered as a pesticide in the U.S. in 1973. Metribuzine is used to selectively control certain broadleaf weeds and grassy weed species on a wide range of sites including vegetable and field crops, turf grasses in recreational areas, and non-crop areas (103). Metribuzine is applied by various methods including aerial, chemigation, and ground application (103, 104).

Environmental fate

Metribuzine, like other triazine and triazinone herbicides, is prone to runoff into surface waters due to its physical and chemical characteristics: water solubility 1.220 mg/L; Koc 41; vapor pressure 1.3 mPa; and soil half-life 30 days (104, 105). The degradation of metribuzine is through photochemical, chemical and biochemical deamination. Aqueous photolysis of metribuzin is rapid with a half-life of <1 day, and this clearly contributes to the half-life of <7 days in natural pond water. Contamination of waters could result from spray and vapour drift, runoff or leaching from treated land, or from accidental spills. Measured environmental concentrations of metribuzine in water are usually low, with maximum concentrations below 1.8 µg/L (106), but modelling studies have indicated that metribuzine can reach concentrations as high as 390 g/L in surface water runoff (104).

Acute toxicity

During the acute exposure of metribuzine fish show increased respiration and loss of movement and coordination. Fish lying on the bottom of the tank and moving in circles, followed by a short excitation stage (convulsions). Necropsy after acute exposure can revealed increased watery mucus on body surfaces, black pigmentation of the skin, and abdominal distention with generalized edema. The body cavity contains transudate, and hyperemia of visceral organs and ascites (26).

Acute toxicity 96hLC50 of metribuzine for fish

Table 5: The effect of atrazine on other fish

Species	Concentration	Exposure	Effects	Reference
Rhamdia quelen Juvenile	2, 10, 100 µg/L	96 hours	↓ CAT, GST, GPx, GR, leukocyte infiltration, hepatocyte vacuolization like steatosis and necrosis areas, leading to raised lesion index levels in all tested concentrations. ↑ free melanomacrophage	(88)
Prochilodus lineatus Juvenile	2, 10 pg/L	24, 48 hours	↓ EROD, ROS, CAT, SOD, GPx, GR, MDA in liver	(89)
Silver catfish (Rhamdia quelen) Juvenile	1.02 mg/L	24 hours	↓ bactericidal activity of the serum, bacteria agglutination, total serum peroxidase activity	(90)
Prochilodus lineatus Juvenile	10 µg/L	14 days	↑ GST, SOD, CAT, LPO	(91)
	25 µg/L		scattered lesions in gill	
Prochilodus lineatus Juvenile	25 µg/L	48 hours	↓ osmolarity	(92)
		14 days	↓ CA; ↑ Na+, Cl-, MRCs in filament epithelium of gill	
Rhamdia quelen Juvenile	0.73 mg/L	96 hours	↓ intracelomatic cells, phagocytic index	(93)
Fathead minnow (*Pimephales promelas*) Adult	0.5, 5.0, 50 µg/L	30 days	↓ production of egg; pathological lesions in testes: granulomatous inflammations, mineralized material in testicular tubules and efferent ducts at rates, variably-sized perinucleolar stage oocytes	(94)
Green Snakehead (*Channa punctata*) Juvenile	4.238 mg/L	5, 7, 10, 15 days	↑ SOD	(58)
	5.3, 10.6 mg/L		↑ SOD, TBARS, CAT	
Rare minnow (*Grobiocypris rarus*) Adult – male	333 µg/L	28 days	↑ HSI, hypertrophy of hepatocytes	(95)
Rare minnow (*Grobiocypris rarus*) Adult	3, 10 µg/L	28 days	lesions in gill including hyperplasia, necrosis in epithelium region, aneurysm and lamellar fusion; lesions in kidney included extensive expansion in the lumen, degenerative and necrotic changes of the tubular epithelia, shrinkage of the glomerulus, increase of the Bowman's space	(96)
Caquetaia kraussii Juvenile	2.5 µg/L	72 hours	hepatocytes lost the cytoarchitecture (the hepatocytes have different diameters and irregular contour); isolated associations between mitochondria and rough endoplasmic reticulum in the cytoplasm	(97)
Rhamdia quelen Juvenile	3.5, 5.25 mg/L Herbimix® (simazine + atzrazine)	96 hours	↑ cortisol	(98)
Goldfish (*Carassius auratus L.*) Juvenile	1 000 µg/L	56 days	↑ 11-KT	(99)
Red drum (*Sciaenops ocellatus*) Larvae	40, 80 µg/L	4 days	↓ growth; behaviour: swam significantly faster, with a higher rate of travel, active swimming speed, hyperactive, swam considerably more convoluted paths compared to control	(100)
Goldfish (*Carassius auratus*) Juvenile	0.5 µg/L	24 hours	↓ sheltering, grouping behavior, burst swimming; ↑ surfacing activity	(101)
Mormyrid fish (*Gnathonemus petersii*) Juvenile	0.5, 5 mg/L	6 hours	breaks in the gill epithelium, which developed into deep pits	(102)

is ranging from units to hundreds milligrams per liter (Tab. 1.).

Effect of metribuzine on fish

The effects of metribuzine on fish physiology have been well-documented. Metribuzine affected hematological, biochemical profile, growth and caused hitopatological changes in tissues (Tab. 6.). During acute poisoning of metribuzin in rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio*), the following clinical symptoms are observed: accelerated respiration, loss of movement coordination, fish lying on their flanks and moving in this position. The subsequent short excitation stage (convulsions, jumps above the water surface, movement in circles) changes into a resting stage and another short-time excitation follows again. In the end, fish fall into damp, moving mainly on their flanks. The respiration is slowed down, and the damp phase and subsequent agony are very long. Fish are produceds of watery mucus on body surfaces, the skin is matt dark in colour and the ventricle expansion. The body cavity contained transudate, and an increased injection of visceral vessels is also obtained (26, 107).

Prometryne

Prometryne (2,4-bis(isopropylamino)-6-methylthio-s-triazine) was the first effective herbicide for several crops, making it a true pioneer herbicide in the methylthiotriazine class of chemistry (112) and was first registered in 1964 by Ciba Crop Protection (113). Prometryne is selective herbicide of the s-triazine chemical family, has been utilized as a pre- or post-emergence controller of annual grasses and broadleaf weeds in a variety of crops, including cotton, celery, pigeon peas and dill. Prometryn's mechanism of action inhibits the electron transport in susceptible species (114). Prometryne application is not permitted in Europe, but is widely used in China (115), Australia, Canada, New Zealand, South Africa, and the United States (28).

Environmental fate

Prometryne is usually soil-applied and relatively water soluble, it tends to accumulate in crops (114). Prometryne binds readily to soils with high clay and organic matter content. Available data indicate that this herbicide is mobile in sandy soils and moderately mobile in sandy loam soils. Its mobility appears to be related to organic content of the soil. Prometryne the lower the organic content, the more mobile prometryne is in soil. Prometryne is adsorbed to a greater extent than most other commercial triazine herbicides (116). Prometryn is a persistent chemical, it is persists in the soil from one to three months. Its soil half-life is 60 days. Following multiple annual applications of the herbicide, prometryne activity can persist for 12-18 months after the last application. It will persist longer under dry or cold conditions which are not conducive to chemical or biological activity. It resists abiotic hydrolysis, direct photolysis, and biodegradation under anaerobic conditions. Its half-life under aerobic conditions is in excess of 270 days (117).

Significant traces of prometryne are documented in the environment, mainly in water, soil, and plants used for human and domestic animal consumption. Maximal environmental concentration prometryne is 0.51 μg/L in the Czech rivers (14). In surface waters of Greece, prometryne has been recorded at concentrations from 0.19 to 4.40 μg/L (118). Prometryne to contaminate the groundwater resources of the Axios river basin in Macedonia, Northern Greece, during 1992–1994 were detected at concentrations occasionally exceeding 1 μg/L (118). In surface water of Western France, remains of prometryne were detected at concentrations from 0.1 to 0.44 μg/L (119).

Acute toxicity

Exposure prometryne to nontarget organisms can result from direct applications, spray drift, and runoff from treated areas. Studies indicate that prometryne poses an acute risk to nonendagered and endangered terrestrial and aquatic plants (113). Prometryne is toxic to fish (Tab. 1.). The most sensitive aquatic organisms are freshwater algae (14).

Effect of prometryne on fish

Although the lethal toxicity of fish to prometryne, have been well-documented, there is a dearth of data on the effects of prometryne on fish physiology.

Table 6: Effect of metribuzine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Bluegill (*Lepomis macrochirus*) Juvenile	9, 19, 38, 75 µg/L	6 weeks	No effects on fish survival and growth	(103)
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	89.3 mg/l Sencor 70 WG (active substance 70% of metribuzin)	96 hours	↓ TP, TAG, AST, NH3, Ca, LACT, ALP, RBC, PCV, lymphocyte coun. ↑ MCH, relative and absolute count of neutrophile granulocytes Revealed mild proliferation of goblet cells of the respiratory epithelium of secondary gill lamellae and hyaline degeneration of epithelial cells of the renal tubules of the caudal kidney.	(107)
Common carp (*Cyprinus carpio*) Juvenile	1.75 mg/L	28 days	↑ RBC, PCV	(108)
Common carp (*Cyprinus carpio*) Juvenile	250.2 mg/L Sencor 70 WG (active substance 70% of metribuzin)	96 hours	↑ GLU, NH3, Ca, monocytes, neutrophile granulocytes, developmental forms myeloid sequence,basophiles. ↓ TP, ALB, GLOB, TAG, LDH, LACT, PHOS, PCV, Hb, MCV, WBC, lymphocyte Revealed hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney.	(26)
Common carp (*Cyprinus carpio*) Embryo - larvae	0.9, 4, 14, 32 mg/L	30 days	↑ GST	(109)
	0.9, 4, 14 mg/L		↑ GR	
	0.9 mg/L		↑ TBARS	
Common carp (*Cyprinus carpio*) Embryo - larvae	0.9, 4, 14, 32 mg/L	30 days	↓ specific growth rate, body weight, length	(110)
	32 mg/L		Diffuse vacuolization of the cytoplasm of hepatocytes, often with compression of nuclei at the periphery of the cells. Monocellular necroses of hepatocytes. Eosinophilia of tubular epithelial cells with coagulation of cytoplasm and desquamation of necrotic cells into the lumen of proximal tubules in the caudal kidney.	
Zebrafish (*Danio rerio*) Juvenile	33, 55 mg/L	28 days	↓ specific growth rate, body weight, length	(111)
	55 mg/L		Moderate dystrophic lesions of hepatocytes, initial cell injury represented by diffuse hydropic to vacuolar degeneration of hepatocytes.	

Only three studies on effects of prometryne on carp physiology have been conducted (Tab. 7.). Chronic exposure has no influence on growth, oxidative stress biomarkers and it has influence on hematological, biochemical plasma indices, antioxidant enzymes and caudal kidney (120-122).

Simazine

Simazine (6-chlor-N2,N4-diethyl-1,3,5-triazin-2,4-diamin) is one of the first compound triazines (a six-membered ring containing three carbon and three nitrogen atoms), was introduced by a Swiss company J. R. Geigy in 1956 and was registered in 1957 (5). From 1990 to 1993 are among the most widely used herbicides in the U.S. Simazine belongs to a group of selective triazine herbicides, is used for a pre- and post-emergence control most weeds field crops as well as in non-crop areas. When applied to the soil is absorbed by leaves and roots, causing inhibition of photosynthesis in whole plants (123). It is biodegradable, is metabolized in plants and soil, both chemical, and microbiological processes (112). It is fairly resistant to physical and chemical dissipation processes in the soil. It is persistent and mobile in the environment (124). Even before 1992 simazine was used to kill submerged (growing in water) weeds and algae in large aquariums, ponds, swimming

Table 7: Effect of prometryne on fish

Species	Concentration	Exposition	Effects on fish	Reference
Common carp (Cyprinus carpio) Embryo - larvae	0.51, 80, 1 200 μg/L	35 days	↓ GR activity	(120)
Common carp (Cyprinus carpio) Juvenile	80 μg/L	14 days	↓ GR in brain, SOD in intestine	(121)
	8, 80 μg/L		↓ SOD in gill, ↑ SOD in brain	
	0.51, 8, 80 μg/L		↑ GR in muscle	
	8, 80 μg/L	30 day	↓ SOD in brain	
	0.51, 8, 80 μg/L		↓ SOD in gill	
	80 μg/L	60 days	↑ CAT in intestine, ↓ CAT liver, SOD in gill	
Common carp (Cyprinus carpio) Juvenile	80 μg/L	30 days	↑ GLU	(122)
	8, 80 μg/L	60 days	↑ GLU, MCH, MCHC, Hb ↓ SW, LACT	
	0.51, 8, 80 μg/L	30, 60 days	↑ CK, ALT, ↓ AST, Ca, Mg, PHOS	
		60 days	Hyaline degeneration of the epithelial cells of caudal kidney tubules	

pools or cooling towers (125). Simazine and plant protection products containing this substance were banned in 2004 by Commission Decision 2004/247/CE. The presence of simazine in the soil–water system in considered an environmental hazard, and, because of its estrogenic effect on various cell lines in laboratory experiments, it has recently become subject to control (6, 126).

Environmental fate

Simazine in soil and groundwater is moderately persistent with an average field half-life of 60 days. Soil half-lives have been reported of 28-149 days (127). Residual activity may remain for a year after application (2 to 4 kg/ha) in high pH soils. Simazine is moderately to poorly bounds to soils (105). Simazine is metabolized in plants and soil, both chemical, and microbiological processes (125). It does, however, adsorb to clays and mucks. It is low water solubility, however, makes it less mobile, limiting it is leaching potential. Simazine has little, if any, lateral movement in soil, but can be washed along with soil particles in runoff. Simazine is subject to decomposition by ultraviolet radiation, but this effect is small under normal field conditions. Loss from volatilization is also insignificant. In soils, microbial activity probably accounts for decomposition of a significant amount of simazine in high pH soils. In lower pH soils, hydrolysis will occur (48).

Simazine can be persistent in aquatic systems, particularly in shallow, well-mixed lakes and ponds (128). Residues may persist up to 3 years in soil under aquatic field conditions. Dissipation of simazine in pond and lake water has been found to be variable, with half-life ranging from 50 to 700 days (105). Slow biodegradation of simazine may occur in water, similar to that observed in soil. Simazine may undergo hydrolysis at lower pH. It does not readily undergo hydrolysis in water at pH = 7 (48). Simazine and its degradation products are detected less frequently than atrazine in the aquatic environment.

Simazine is the second most commonly detected pesticide in surface and ground waters in the U.S., Europe, and Australia. Simazine, and its major degradation products (deisopropyl atrazine and diamino chlorotriazine), have been extensively monitored in 20 counties in California with concentrations ranging from 0.02 to 49.2 μg/L (129, 130). Simazine levels can reach values, up to 5.0 μg/L in Europe rivers (131-134).

Table 8: Effect of simazine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Seabream (*Sparus aurata*) Larvae	4.5 mg/L	72 hours	Cellular alterations related to loss of cellular shape in hepatocytes, lipid inclusions, focal necrosis and abundant nuclear pyknosis in the hepatocytes.	(136)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	90 days	↑ mucus production during the experiment, Hyperplasia of epithelial cells of seconadary lamellae, slight necrosis	(137)
Goldfish (*Carassius auratus*) Adult	50 µg/L ∑ atrazine +simazine + diuron + isoproturon	4, 8, 12 weeks	↑ plasma lysozyme aktivity; production of O2.– in spleen, kidney; SOD in spleen and liver; ↓ antibody titre, CAT in liver, spleen, kidney	(138)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	90 days	↓ AChE in brain and muscle	(139)
Rhamdia quelen Juvenile	16.6%, 33% 50% 96h LC50 hatrazine + simazine (Herbimix™)	96 hours	Decreased capacity in exhibiting an adequate response to cope with stress and in maintaining the homeostasis, with cortisol level lower than that in the control fish	(140)
Common carp (*Cyprinus carpio*) Juvenile	4, 20, 50 µg/L	28 days	↑ PCV, lymphocytes, developmental phases –myeloid sequence, GLU, LDH, CK, CREA; ↓ MCHC, neutrophil granulocytes bands, NH3, AST Decline in hematopoietic tissue in caudal kidney; steatosis, hyperaemia, and necrosis in liver	(141)
Common carp (*Cyprinus carpio*) Juvenile	45 µg/L	15, 30, 45, 90 days	No efect on muscle LACT, LDH	(142)
			↑ mucus hyperproduction in gills and skin; No efect on MDA and GSH	(143)
		90 days	↑ PCV, necrotic areas in hematopoietic and excretory tissues of the kidneys; Isolated necrotic areas in liver	(144)
Rhamdia quelen Juvenile	16.6% 96h LC50 hatrazine + simazine (Herbimix™)	96 hours	↑ plama cortisol	(145)
Zebrafish (*Danio rerio*) Juvenile	60 µg/L	28 days	Hypertrophy, hyperplasia of epietelial gill cells with lamellar fusion. Initial cell injury represented by swelling and hydroscopic vacuolar degeneration of hepatocytes). Coagulation of the apical part of the cytoplasma of epithelial cells of the renal tubules	(146)
Common carp (*Cyprinus carpio*) Embryo - larvae	60 µg/L	35 days	Alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules	(147)
	0,6, 3 mg/L		↓ growth; alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules	
Common carp (*Cyprinus carpio*) Juvenile	0.06 µg/L	90 days	↑ ALP; ↓ WBC; hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	(148)
	1, 2 µg/L		↑ HSI, ALP, AST; ↓ WBC; hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	
	4 µg/L		↑ HSI, TP, ALB, AST, ALP; ↓ WBC hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney	

Common carp (Cyprinus carpio) Juvenile	0.06 µg/L	28 days	↑ GSH in liver;	(149)
		60 days	↑ CAT in muscle, GSH in liver	
	2 mg/L	14 days	↑ SOD in muscle; CAT in muscle, liver; GSH in liver; ↓ GPx in liver	
		28 days	↑ SOD in muscle CAT in muscle, liver; GSH in liver; ↓ GPx in liver	
		60 days	↑ ROS in liver; GSH in liver, brain; ↓ SOD in muscle; CAT in muscle, liver;	
	4 mg/L	14 days	↑ CAT in liver; SOD in muscle; GSH in liver, brain; ↓ GPx in liver	
		28 days	↑ ROS in liver; SOD in muscle; GSH in liver, brain; ↓ GPx in liver	
		60 days	↑ ROS in muscle, brain, liver; GST in brain; ↓ GST and GPx in liver, SOD in muscle; CAT in brain, liver, muscle	

Acute toxicity

Simazine was identified as relevant a study of the prioritization of substances dangerous to the aquatic environment in the member states of the European Community (10). Lethal acute toxicity for fish is ranging from units to hundreds milligrams per liter (Tab. 1.).

Effect of simazine on fish

The effects of simazine mainly on carp physiology have been well-documented in laboratory studies. Chronic exposure of simazine has influence mainly on growth, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, and caused histopathological changes in gill, liver and kidney (Tab. 8.). Simazine has been recently reported as suspected endocrine disruptors, it is also known to cause multiple types of cancers (135).

Terbuthylazine

Terbuthylazine (N-tert-butyl-6-chloro-N'-ethyl-1,3,5-triazine-2,4-diamine) was registered in the United States in 1975 (150). Terbuthylazine is herbicide that belongs to the chlorotriazine family, is used in both pre- and post-emergence treatment of a variety of agricultural crops and in forestry (118). Terbuthylazine have very similar chemical structure to atrazine. The difference is only iso-butyl and tert-butyl substituent on the amino group. The minimum difference in structure affects the decomposition reactions of these substances in the environment that led to a ban on atrazine in the European Union. The EU had more stringent drinking water standards caused farmers to shift from atrazine to terbuthylazine. Terbuthylazine is used as a substitute for atrazine since the end of 2006 (151). Terbuthylazine breaks down much more rapidly than atrazine in both soil and water, and is therefore believed less likely to contaminate drinking water (152).

Environmental fate

Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions (150). Terbuthylazine is a slightly basic, slightly water soluble triazine herbicide or algacide which adsorbs to soil organic matter. Degradation of terbuthylazine in natural water depends on the presence of sediments and biological activity (124). Under laboratory conditions, aquatic photolytic half-lives ranged from around 3 hours (attenuated) to a more realistic 1.5-5 days under more usual test conditions that seem to be reflected in the recommended use pattern. Usually, the main degradation product was hydroxy-terbuthylazine, although with an attenuator N-dealkylation is favoured. Laboratory studies in soils (sandy loam) gave half-lives of 73-138 days at 20-25 °C, but this extended to 456 days at 10 °C, with hydroxy-terbuthylazine and desethyl-terbuthylazine as the

Table 9: Effect of terbuthylazine on fish

Species	Concentration	Exposition	Effects on fish	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	35.1, 42.9, 45.8 µg/L	7 days	↓ EROD, UDPGT	(159)
European sea bass (*Dicentrarchus labrax* L.) Juvenile	3.55, 5.01, 7.08 mg/L	24 hours	↑ RCs in gills, intestine, kidney histopatological examination displayed cellular and/or ultrastructural alterations in all the organs examined. In the gills necrosis, lamellar and cellular oedema, epithelial lifting, telangectasia, and fusion of secondary lamellae were encountered. The liver presented myelin-like figures, cytoplasmic rarefaction and acute cell swelling of hepatocytes. The renal tubular epithelial cells, exhibited 'blebs'.	(158)
		48 hours	↑ RCs in gills, intestine histopatological examination displayed cellular and/or ultrastructural alterations in all the organs examined. In the gills necrosis, lamellar and cellular oedema, epithelial lifting, telangectasia, and fusion of secondary lamellae were encountered. The liver presented myelin-like figures, cytoplasmic rarefaction and acute cell swelling of hepatocytes. The renal tubular epithelial cells, exhibited 'blebs'.	
Common carp (*Cyprinus carpio*) Juvenile	550 µg/L	91 days	↑ TAG, ALB, Na, TP, EC, FRAP ↓. MCHC, MCH, MCV, AST, P	(160)
	60 µg/L		↑ TAG, ALB ↓. MCH, MCV, AST, P	
	380 ng/L		↑ HSI, CF, TAG, TP	
Common carp (*Cyprinus carpio*) Juvenile	13.0 mg/L Gardoprim Plus Gold 500 SC (corresponding to 2.25 mg/L terbuthylazine and 3.75 mg/L S-metolachlor	96 hours	↑ GLU, AST, NH3, LDH ↓ lymphocyte counts, WBC, PCV, PHOS, TAG, chlorides lesions in gills and liver	(161)
Common carp (*Cyprinus carpio*) Embryo - larvae	520 µg.L^{-1}	30 days	↑ GR	(109)
Zebrafish (*Danio rerio*) Juvenile	400 µg/L	28 days	↑ GST	(162)
	700 µg/L		↑ GR, GST, pathological changes in the liver	
	1000 µg/L		↑ GR, GST, TBARS, pathological changes in the liver	
Common carp (*Cyprinus carpio*) Embryo - larvae	520, 820 µg/L	30 days	↓ specific growth and body weight, delay in development, mild leisons in liver including diffuse formation of smal round to oval vacuoles in the cytoplasma of hepatocates	(163)
Common carp (*Cyprinus carpio*) Juvenile	3.3 mg/L	24 hours	↑ GLU, AST, ALT, natrium, chlorides, phosporus, Ca, circulation disorders in gills represented by abundant presence of capillary aneurysms in gill filaments and a local hyperplasia of respirátory epithelium	(164)
Common carp (*Cyprinus carpio*) Embryo - larvae	0.0029, 0.07, 1.4, 3.5 mg/L terbuthylazine-2-hydroxy	26, 35 days	↓ SOD, specific growth and body weight	(165)
	1.4, 3.5mg/L terbuthylazine-2-hydroxy	35 days	damage to caudal kidney tubules, delay in development	

Table 10: Effect of terbutryne on fish

Species	Concentration	Exposition	Effects on fish	Reference
Rainbow trout (*Oncorhynchus mykiss*) Juvenile	28.3 29.2, 32.6 μg/L	7 days	↓ EROD, UDPGT	(159)
Seabream (*Sparus aurata*) Larvae	2.5 mg/L terbutryn+triasulfuron	72 hours	cellular alterations related to loss of cellular shape of hepatocytes and intense nuclear pyknosis in the hepatocytes	(175)
Zebrafish (*Danio rerio*) Juvenile	0.6 mg/L	28 days	↓ specific growth; wieght, damage to tubular system of kidneys	(176)
Common carp (*Cyprinus carpio*) Juvenile	2, 20, and 40 μg/L	28 days	↑ RBC, NH3, AST, LDH, CK, LACT ↓ MCV, MCH, CK Diffused steatosis of the liver - the loss of cellular shape and the presence of lipid inclusions in hepatic cells; damage to caudal kidney tubules	(177)
Common carp (*Cyprinus carpio*) Juvenile	0.2, 2 μg/L	90 days	↑ RBC, MCHC, neutrophil granulocyte bands, GLU, AST, LDH, LACT, TBARS in brain, liver; CP in brain, gill; SOD in liver, brain ↓ WBC, MCV, CK, Mg, GR in liver, intestine	(178)
	0.02 μg/L		↑ TBARS in brain, liver, SOD in liver ↓ GR in liver	
Common carp (*Cyprinus carpio*) Embryo - larvae	2 mg/L	30, 36 days	↓ CF	(179)
	0.2, 2 mg/L		delay in development	
	0.02, 0.2, 2 mg/L		Alteration of tubular system in caudal kidney included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomeruli	
	0.00002, 0.02, 0.2, 2 mg/L		↓ mass and total length; damage to caudal kidney tubules	

main degradation products (153). Terbuthylazine photo-degrades in water this is likely to be the main degradation pathway. The fate of residues in aerobic and anaerobic aquatic conditions is similar. The major metabolites of terbuthylazine are the de-chlorinated and N-dealkylated products, which are more mobile than the parent, and exhibit some herbicidal activity when they retain the chlorine atom on the triazine ring plus one alkyl group (152, 153).

Terbuthylazine levels can reach values up to 2.9 μg/L in Europe rivers (40, 154, 155). The groundwater situation in different countries was surveyed by the French Ministry of Agriculture and Fisheries. In Germany and Sweden 22 out of 3204 samples and 6 out of 230 samples were positive for terbuthylazine (above 0.1 μg/L), respectively (156).

Acute toxicity

The ecotoxicity profile of terbuthylazine is typical for a herbicide, with toxic effects mostly apparent towards plants/algae. However, terbuthylazine shows slight toxicity towards fish and shellfish, and variable toxicity towards aquatic crustaceans, from very highly toxic to practically non-toxic (124). Standard toxicity tests with various fish species as nontarget organisms revealed LC50 values between 4.6 and 66 μg/L (Tab. 1.). As a consequence, terbuthylazine might be considered as a moderately or slighty toxic. The acute exposure to terbuthylazine, however, leads to significant alterations of the average swimming velocity on the fish. After a nonuniform initial phase of swimming irritation, an increase in motility can be observed. With every exposure tested, this hyperactivity exceeded any preexposure motility (157).

Effect of terbuthylazine on fish

Exposure to terbuthylazine affected on growth, oxidative stress biomarkers, hematological, biochemical plasma indices, antioxidant enzymes, detoxification enzymes and caused the histopatological changes in gill, liver, intestine and kidney (Tab. 9.). Fish during the terbuthylazine intoxication showed uncoordinated swimming and hyporeflexia increasing (158).

Terbutryne

Terbutryne (N2-tert-butyl-N4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) was used as a selective pre- and early post- emergence controll agent of most grasses and many annual broadleaved weeds for a variety of crops, such as cereals, legumes, and tree fruits. It is also used as a herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs, and fish ponds (166, 167). Large quantities of terbutryne have been used since the mid-1980s (168). Terbutryne and plant protection products containing this substance were banned in 2005 by Commission Decision 2004/247/CE.

Environmental fate

Terbutryne degrades slowly, with a half-life of 240 and 180 days in pond and river sediments, respectively (169). Its tendency to move from treated soils into water compartments through water runoff and leaching has been demonstrated, and residual amounts of terbutryne and its metabolites have been found in drinking water and industrial food products long after application (170). The application of terbutryne has been banned in many countries because it has the potential to bioaccumulate in organisms, but it has been still detected in water environment (171). The highest concentration reported in surface water in the Weschnitz River, Germany, at a maximal concentration of 5.6 μg/L from September 2003 to September 2006 (172). Terbutryn was also detected in Mediterranean coastal waters at a concentration of 5-184 ng/L (173).

Acute toxicity

Acute toxicity 96hLC50 of terbutryne for fish is ranging from units of milligrams per liter. Terbutryne is toxic to fish (Tab. 1.).

Effect of terbutryne on fish

The effects of terbutryne mainly on carp, zebrafish and rainbow trout, physiology have been documented in laboratory studies. Chronic exposure of terbutryne has influence mainly on growth, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney (Tab. 10.). The results demonstrate that the terbutryne accumulated to a somewhat greater extent in the viscera (liver, intestine, and pyloric caeca) than in the muscle tissue of the carp and trout during exposure (169, 174). Bioconcentration factors (BCFs) of terbutryne for fish were estimated 312 (169).

Conclusion

Triazines are predominant class of herbicide. They are most frequently detected pesticide in aquatic environment. Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and they are included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. All of above cited seven triazines are banned or severely restricted in EU (180). Acute toxicity was assessment on 28 fish species. Toxic effect of triazine has influence mainly on growth, early development, oxidative stress biomarkers, antioxidant enzymes, hematological, biochemical plasma indices, caused histopatological changes in liver and kidney. Investigation of triazine and their metabolites properties in connection with environment, chronic effects and potential bioaccumulation must continue thoroughly. Research on non-target species should be really detailed and should continue because as can be seen in the previous text, triazines are able to cause pathological changes in fish. We assume

that triazines and their metabolites have similar effects on other non-target organisms as to have on fish. As shown some studies on crayfish (181-183). It is necessary to focus on the research of triazines metabolites using new molecular techniques and gene expression.

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UČINEK TRIAZINSKIH HERBICIDOV NA RIBE: PREGLED

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Povzetek: Onesnaževanje okolja je svetovni problem, ki povzroča vse večjo zaskrbljenost in je posledica različnih človekovih dejavnosti povezanih z industrijo in kmetijstvom. Triazinski herbicidi so med najpogosteje uporabljenimi pesticidi. V zadnjem času vse bolj naraščata zavedanje in zaskrbljenost zaradi njihove široke uporabe, saj so ostanki in presnovki triazinov zelo obstojni in se kopičijo v različnih delih okolja. Triazini so bili zaznani tudi v vodnih ekosistemih, v pitni vodi in podzemnih vodah ter tudi v ribah. Zato je uporaba določenih triazinskih pesticidov v evropskih državah že prepovedana. Osem s-triazinov je bilo uvrščeno v študijo za pripravo prednostnega seznama snovi, nevarnih za vodno okolje v državah članicah Evropske unije in so že vključeni v prednostni seznam onesnaževalcev okolja v Evropski unije in ZDA (*European Union Priority Pollutants List* in *U.S. Environmental Protection Agency's List*). V preglednem članku je predstavljeno trenutno poznavanje stanja ostankov triazina v vodnem okolju in njihovi strupeni učinki na ribe. Na osnovi pregleda dosedanjega poznavanja problematike smo opredelili glavne vrzeli v trenutnem znanju in nekatere usmeritve za prihodnje raziskave. Pregled vsebuje vpliv sedmih najpogosteje odkritih triazinov v vodi (ametrin, atrazin, metribuzine, prometrin, simazin, terbutilazin in terburine) na fiziologijo rib in njihovo akutno strupenost. Toksični učinki triazinov vključujejo vpliv na rast rib, njihov zgodnji razvoj, oksidativni stres in izražanje antioksidantnih encimov, pa tudi na krvne in biokemične parametre v plazmi ter na histopatološke spremembe v jetrih in ledvicah rib.

Ključne besede: triazini; ribe; strupenost; biokemični profil; hematologija; histologija

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Original Scientific Article

APPLICABILITY ASSESSMENT OF A STANDARDIZED MICROSATELLITE MARKER SET IN ENDANGERED BUSHA CATTLE

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Summary: This study is focused on evaluating 12 microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824) as recommended by International Society of Animal Genetics (ISAG) for paternity testing in Busha cattle in Serbia. A herd of 47 heads of Busha cattle were sampled for blood. The number of alleles ranged from 6 (ETH10) to 16 (TGLA122), with a mean value of 9.5. Total number of alleles at all 12 analyzed loci was 114. PIC (Polymorphism Information Content) values ranged from 0.513 (in BM1818) to 0.905 (TGLA53). Power of exclusion (PE) for single markers ranged from 0.228 (BM1818) to 0.607 (BM2113) and the power of discrimination (PD) from 0.75 (BM1818) to 0.96 (TGLA227). The combined power of exclusion and discrimination was very high (0.999 and 0.995 respectively) when all 12 markers were used in combination. Cervus software performed 96% successful paternity assignments. These results show that the 12 markers set recommended by ISAG can be used with high confidence for forensic purposes in Busha cattle.

Key words: microsatellite; paternity testing; buša; busha

Introduction

According to FAO and UNEP data (1), about one third of the world's recognized 5000 livestock and poultry breeds are endangered but in both developed and developing countries they represent a unique resource to meet present and future breeding objectives (2). Busha (also Buša or Buscha), is a collective term for small, robust and long-living cattle, the withers' height at around 100 cm (3). Busha cattle are autochthonous in the Balkan area. Being a mixed type of cattle with relatively low productive traits, Busha has been slowly pushed into extinction as farming became more intensive and demanding. Today this breed is classified as endangered by the Republic of Serbia Law on Animal Husbandry (4). Still, busha population in the Republic of Serbia has diminished in past years. One of the larger heards of busha cattle still exists in the mountain region of Dimitrovgrad, in the south-east of the Republic of Serbia (geographic coordinates 43°01′N 22°47′E). The heard was founded in 2005 and since then the cattle was managed under natural breeding conditions. Animals in this heard originate from

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Bujanovac, Trgoviste, Bosilegrad, Sjenica and Tutin areas of the Republic of Serbia. Several color varieties are represented in the heard: gray, dark-gray, black, yellow and gray with tiger stripes.

Accurate parentage identification is essential in establishing a breeding program tailored to conserve genetic variability in endangered cattle breeds. Conservation strategies can be categorized as either conserving animals in-situ (in the environment in which they were developed), or ex-situ (in all other cases). The latter can be further divided into ex-situ *in vivo* conservation and cryogenic storage (5). Since *in vivo* conservation strategies include pedigree recording as an integral step it is clearly important for conservation models that all identified genetic relationships are correct. Given that minimization of the loss of genetic variation is equivalent to minimization of the rate of inbreeding in a population, the rate of increase of inbreeding (ΔF, F representing the coefficient of inbreeding) is the most important parameter in programs that maintain genetic diversity (5). Previous studies show that introduced errors in paternity testing lead to changes in inbreeding coefficient in cattle populations (6).

Traditionally, pedigree verification in cattle was based on blood groups and biochemical polymorphism analyses. Pedigree data are confirmed based on antigens of 12 blood group systems using over 80 test sera (7). However, a high frequency of incorrect cattle paternity was obtained using traditional markers. In addition, blood typing cannot be done retrospectively, e.g. after a sire is dead (8).

Since first described in cattle by Fries et al (9) microsatellites have been used increasingly in population genetics studies and paternity analyses in cattle. To date, there are several thousands of microsatellite markers described in cattle, a great number of which have been proven informative for parentage testing in a large number of cattle breeds. Accessibility of thousands of microsatellite markers for cattle resulted in the creation of different only partly overlapping sets of markers used for parentage analysis (8). In 2006, International Society for Animal Genetics-ISAG (10) recommended 9 microsatellite loci (BM2113, BM1824, SPS115, TGLA227, TGLA126, TGLA122, ETH10, ETH225 and INRA23) as a minimum set of markers for cattle parentage testing. In the current standard set of 12 microsatellites, additional three loci (ETH3, TGLA53 and BM1818) were included in a standard panel for routine testing (11).

To determine the degree of usefulness of microsatellites for parentage testing in a certain population, the number and frequency of their alleles must be evaluated (12). To date there is no information available on applicability of microsatellite markers for paternity testing in Busha cattle breed, despite the fact that Busha is considered a valuable genetic resource, protected under the Law as an endangered autochthonous breed.

In this study a set of 12 microsatellites (BM2113, BM1824, SPS115, TGLA227, TGLA126, TGLA122, ETH10, ETH225, INRA23, ETH3, TGLA53, and BM1818) as recommended by ISAG was evaluated for their use for paternity testing and pedigree verification in an autochthonous breed of cattle – Busha.

Materials and methods

Twelve microsatellites used in this study are recommended by ISAG for cattle paternity testing. Blood samples were taken from 47 heads of Busha cattle. The samples originated from a single heard of animals from south-eastern part of the Republic of Serbia, the region of Dimitrovgrad (43°01′N 22°47′E). Animals sampled were maintained under natural mating conditions. Taking care of the representativeness of sample, animals included in the study were unrelated two generations in the past. The information on the method of reproduction and relatedness of animals was obtained in an interview with the owner. Samples of blood were collected from the coccygeal vein. Genomic DNA from blood was isolated following standard protocol for organic extraction of nucleic acids (13), and kept frozen at –18°C until further processing. Microsatellites were amplified using the "Bovine Genotypes Panel 1.2" (Thermo Fisher Scientific Inc.) in a single multiplex reaction as recommended by FAO (14). The reactions were carried out according to the manufacturer recommendations. The reactions were performed in a programmable thermal cycler MultiGene Gradient (Labnet International Inc.). The fluorescent labeled PCR products were submitted to fragments analysis by capillary electrophoresis, with an automated sequencer ABI PRISM 310 (Applied Biosystems), using the GeneScan-350 ROX® Size Standard (Applied Biosystems), according to the

manufacturer's specifications. Results were read and interpreted using GeneScan® and Genotyper® software, respectively.

Standard statistical procedures were used to assess the informativeness of selected microsatellite markers. The number of alleles (nA), frequency of the most frequent allele (FNA), observed and expected heterozygosity (Ho and He), polymorphism information content (PIC), power of discrimination (PD) and power of exclusion (PE) were calculated for each microsatellite marker. Combined power of discrimination (CPE) and combined power of exclusion (CPE) were calculated for the whole set of studied markers (15, 16). Allele frequencies, PIC, PD and PE were determined by the PowerStatsV12 freeware, Promega Corporation, USA (17). Observed and expected heterozygosity as well as calculations for Hardy-Weinberg Equilibrium (HWE) were performed in Arlequine ver. 3.1 (18) according to Guo and Thompson (19).

To additionally confirm the applicability of the marker set, a simulation of parentage analysis was performed in CERVUS software (20) using the following assumptions: the number of candidate fathers was 46 and proportion of the sampled fathers was 1, since we assume that all the sires were sampled within this population; proportion of loci typed was set to 90% to account for missing or unreadable data, and proportion of loci mistyped was set to 1% to account for eventual mutations and/or mistakes in genotyping. Minimal number of loci typed was set to 9 given that our dataset had missing data in 3 loci. Finally, 100 000 offspring assignments were done.

Results

Informativness of the analyzed marker set assessed by basic diversity indices and forensic parameters is shown in Table 1 and Table 2, respectively.

The results obtained from Cervus software analysis showed that 96% of offsprings can be assigned to a certain father within the population at the strict confidence level of 95%. More precisely, 4452 out of 100 000 assignments were unsuccessful.

Results show high applicability of the tested marker set for individual identification and paternity verification in Busha cattle.

None of the markers deviated significantly from Hardy-Weinberg equilibrium.

Discussion

This study was focused on evaluation of a microsatellite set of 12 markers recommended by ISAG for their applicability in forensic science. Although Busha cattle breed is considered endangered in several countries and definite measures are being employed with the aim of population restitution, data on performance of microsatellite markers in parentage testing and individual identification is not available for this breed. When assessing a certain set of markers for forensic purposes, several guidelines should be followed: the markers should be characterized by high polymorphism, well-balanced frequency of alleles, PIC, H and PE values exceeding 0.5, high electrophoretic separation of alleles and repeatability of results during sizing (21). Further, ideally markers should be amplifiable in a single multiplex reaction to facilitate work and provide a streamlined analysis.

The marker set recommended by ISAG consists of 12 markers distributed on 12 different autosomes. The set is amplified in a single reaction, and detected through three different channels, therefore not requiring a large amount of manual labor in preparation and results reading.

Overall all markers showed robustness in amplification, as the percentage of missing data in the tested population was 1.99%.

However, all of the markers consist of dinucleotide repeats. This could influence the reliability of data obtained if two or more alleles in a genotype differ by a single repeat.

In the tested Busha population, the number of alleles ranged from 6 (ETH10) to 16 (TGLA122), with a mean value of 9.5. This is a relatively high value when compared to similar studies in different cattle breeds. In 2010, Stevanovic et al. (8) tested a marker set of 11 microsatellites for forensic purposes in YU Simmental cattle showing a mean value for number of alleles to be 8.273. The number of alleles was higher in our study in 10 out of 11 markers tested. When compared to findings of Simčić et al. (22), average number of alleles was higher both for Slovenian Cika and Croatian Busha cattle where this value was 8 and 5, respectively, although a different Busha population was sampled and a smaller number of animals was tested. In a study on Polish cattle breeds by Radko et al. (23) the average number of

Table 1: Forensic parameters

Locus	PIC	PE	PD
TGLA227	0.857	0.461	0.96
BM2113	0.821	0.607	0.95
TGLA53	0.905	0.310	0.95
ETH10	0.668	0.456	0.86
SPS115	0.672	0.456	0.87
TGLA126	0.685	0.529	0.88
TGLA122	0.812	0.422	0.94
INRA23	0.789	0.379	0.93
BM1818	0.513	0.228	0.75
ETH3	0.691	0.358	0.88
ETH225	0.735	0.389	0.91
BM1824	0.765	0.329	0.92
		CPE=0.999	**CPD=0.995**

PIC – Polymorphysm Information Content; PE – Power of Exclusion; PD – Power of Discrimination; CPE – Combined Power of Exclusion; CPD – Combined Power of Discrimination

Table 2: Diversity indices

Locus	N	nA	Ho	He	AR	G-W	FNA
TGLA227	86	13	0.72093	0.88044	26	0.48148	19.8%
BM2113	92	10	0.80435	0.84854	40	0.24390	27.2%
TGLA53	78	15	0.61538	0.92374	30	0.48387	14.1%
ETH10	92	6	0.71739	0.72432	12	0.46154	35.9%
SPS115	92	7	0.71739	0.72838	14	0.46667	37.0%
TGLA126	92	7	0.76087	0.73435	14	0.46667	40.2%
TGLA122	92	16	0.69565	0.83564	38	0.41026	34.8%
INRA23	90	10	0.66667	0.82247	18	0.52632	27.8%
BM1818	92	7	0.54348	0.56450	20	0.33333	62.0%
ETH3	92	8	0.65217	0.73316	18	0.42105	44.6%
ETH225	92	7	0.67391	0.77783	14	0.46667	34.8%
BM1824	92	8	0.63043	0.79814	40	0.19512	37.0%
Mean	90.1670	9.5	0.6832	0.7810	23.667	0.41307	
s.d.	4.2180	3.398	0.0693	0.0940	10.782	0.10271	

N – Number of gene copies; nA – Number of Alleles; Ho – Observed heterozygosity; He – Expected heterozygosity; AR – Allelic range; G-W – Garza-Williamson index statistic; FNA – Frequency of the most frequent allele

alleles is reported as 118 loci in 11 microsatellite markers in five breeds. Compared to these results the mean number of alleles in Busha cattle is higher than in each of Polish breeds individually. The most polymorphic marker with 16 alleles is TGLA122, which concurs with findings of Radko et al. (23) where 16 alleles were found in total in all five breeds and Simčić et al. (22) where this marker was most polymorphic in Cika cattle with a total of 10 alleles.

When compared to different studies where several strains of busha cattle were analysed with a larger microsatellite set, in this study the average number of alleles was relatively high – 9.5 as compared to 8.52 found in Croatian Busha (24). Further the value found in this study was higher than values found in other Busha strains such as: 8.76 (Red Metohian Busha), 8.17 (Illyrian Mountain Busha), 7.69 (Macedonian Busha), 7.61 (Gray Gacko Busha) or 7.57 (Illyrian Lowland Busha) (3).

Allelic range was widest in BM2113 and BM1824 where alleles spanned 40 base pairs (bp), whilst was relatively wide in TGLA122, as expected given the high number of alleles. Frequency of the most frequent allele (FNA) was the highest in marker BM1818 and was calculated to 62%. The same allele exhibited lowest values across the marker set not only for He and Ho, but also for PIC.

BM2113 marker showed the highest value for observed heterozygosity, but TGLA53 showed the highest value for expected heterozygosity. Both markers showed very high PIC values, TGLA53 being the marker with the highest PIC value within this marker set. Similar results were obtained in Red-and-White cattle breed for TGLA53 marker (23).

PIC values in Busha cattle population ranged from 0.513 (in BM1818) to 0.905 (TGLA53). The marker with the highest PIC value was TGLA53. This marker has a very high number of alleles (fifteen) with relatively wide distribution (30 bp) and a relatively low frequency of the most frequent allele – 14.1%. All these attributes distinguish TGLA53 as one of or the most informative marker in this set. When compared to different breeds of Polish cattle, PIC value for this marker is quite high. Mean PIC value across the marker set was 0.743. These values are higher than those found in many high-production breeds such as YU Simmental cattle (8), Czech Pied cattle, Slovakian Pied cattle (25) and Simmental cattle from Poland (26).

In this study, power of exclusion (PE) for single markers ranged from 0.228 (BM1818) to 0.607 (BM2113) and the power of discrimination (PD) from 0.75 (BM1818) to 0.96 (TGLA227). Most importantly, the combined power of exclusion and discrimination was very high (0.999 and 0.995 respectively) when all 12 markers are used in combination.

Given that the input parameters of the Cervus software mimic the real state of the tested population, and that 96% of paternity assignments were successful, the 12 markers tested proved sufficient for accurately identifying parentage even when taking missing data into account. These results further confirm that the marker set is highly applicable for paternity testing in Busha cattle.

Even though all of the markers within this marker set are consisted of dinucleotide repeats, markers have high PIC, PE and PD values and the whole set performed excellent for forensic purposes in the tested population.

In conclusion, the results of this study show that the analyzed marker set can be used with high confidence in parentage testing and individual identification in Busha cattle.

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OCENA UPORABNOSTI STANDARDIZIRANIH MIKROSATELITSKIH OZNAČEVALCEV PRI OGROŽENI VRSTI GOVEDA BUŠA

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Povzetek: V študiji smo želeli preveriti uporabnost 12 mikrosatelitskih označevalcev (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824), ki jih priporoča Mednarodno združenje za živalsko genetiko (ISAG), za testiranje očetovstva pri govedu buša v Srbiji. Analizirali smo vzorce krvi 47 živali. Skupno število alelov je bilo med 6 (ETH10) in 16 (TGLA122), s srednjo vrednostjo 9,5. Skupno število alelov vseh dvanajstih analiziranih lokusov je bilo 114. PIC (*angl. Polymorphism Information Content*) vrednosti so bile med 0,513 (v BM1818) in 0,905 (TGLA53). Moč izključitve (PE) se je za posamezne označevalce gibala od 0,228 (BM1818) do 0,607 (BM2113) pri moči diskriminacije (PD) od 0,75 (BM1818) do 0,96 (TGLA227). Skupna moč izključevanja in diskriminacije je bila zelo visoka (0,999 in 0,995), ko smo uporabili kombinacijo vseh 12 označevalcev. S programom Cervus smo opravili analizo staršev in s 96% uspešnostjo določili očetovstvo. Ti rezultati kažejo, da se 12 označevalcev, ki jih ISAG priporoča, lahko z visokim zaupanjem uporablja za forenzične namene pri govedu buša.

Ključne besede: mikrosateliti; testiranje očetovstva; govedo buša

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Original Scientific Article

INFLUENCE OF SEX, SLAUGHTER WEIGHT AND SEASON ON CARCASS CHARACTERISTICS OF LIKA PRAMENKA LAMBS RAISED UNDER SEMI-EXTENSIVE PRODUCTION SYSTEM

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Summary: The aim of the study was to evaluate effects of sex, slaughter weight and season on carcass characteristics of Lika Pramenka lambs (30 males and 30 females). All lambs were reared traditionally on natural pastures within two seasons and were slaughtered at the age of five months. Male lambs had deeper chests and longer hind legs than females. Furthermore, male lambs had higher proportion of shoulder, hind legs, shank, head, and lower proportion of rack. Female lambs had lower bone and muscle proportions and higher fat proportions in individual cuts. Slaughter weight significantly affected hot carcass weight, dressing percentage, stomach with intestines, lungs with hearth, skin with feet, chest depth, proportion of belly, shank, kidney knob and channel fat. Season affected all slaughter traits, linear measurements and non-carcass components (except spleen and skin with feet). Furthermore, season affected proportion of neck, rack, hind legs, shank, belly, flank, and fat and bone proportions in the carcass i.e. fat proportion in shoulder, rack, loin, hind legs and belly, and muscle proportion in rack and belly. In order to provide more uniform lambs at the market it is necessary to assure typical feeds in unfavourable seasons and slaughter lambs at narrower weight ranges. Since typical and "natural" meat products are gaining popularity it is necessary to conduct more detailed studies with sensory evaluation and consumers included.

Key words: carcass composition; linear measurements; non-carcass components; slaughter traits

Introduction

The Lika Pramenka sheep belongs to a group of indigenous breeds of Pramenka incurred in mountainous region of Lika and Gorski Kotar (Croatia). It is traditionally reared under semi-extensive production system. In the summer period, animals are free-ranged at natural pastures and at the night in the stable. During the winter period animals are kept in stable, fed with hay and occasionally supplemented with concentrate feed. Lambing season occurs during the spring period, from February till the end of May. According to the Annual report of the Croatian Agricultural Agency (3), the estimated population of Lika Pramenka breed is 30,000 individuals, with 8,714 animals under selection control. Despite the fact that is a multi-purpose breed, in the last decade Lika Pramenka breed is primarily reared for meat production. Lambs are slaughtered at the age of approximately 5 months achieving live weight from 25 to 30 kg i.e. carcass weight between 12 and 15 kg. According to native consumers' opinion, this meat is considered as having high edible quality (17).

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In recent years, with increasing emphasis on sustainable farming systems, the use and exploitation of indigenous breeds have elicited particular attention (7). The increasing demand for healthy, safe meat products is stimulating market interest in extensive production as an important part of a sustainable system (13) which can produce a carcass of a superior quality and in accordance with the demands of consumers (15). In contrast to lighter lambs and according to our knowledge from literature reports, carcass characteristics of medium-sized lambs raised in semi-extensive or extensive production system recently has not been widely studied.

Therefore, this study aims to determine the main factors (sex, slaughter weight and slaughtering season) affecting carcass characteristics of Lika Pramenka lambs raised in traditional semi-extensive production system.

Material and methods

The study was carried out during the slaughtering season in years 2010 and 2011 on a single family farm placed in the southern part of the mountain region of Croatia (Gospić). According to Croatian Bureau of Statistics, average monthly air temperatures during the slaughtering season in 2010 ranged from 0.7 to 17.9 °C and in 2011 from 0.2 to 18.4 °C. Average precipitation rates during the slaughtering season in 2010 ranged from 98.4 to 158.6 mm and in 2011 from 18.0 to 57.6 mm. Lambs were born at the beginning of March and kept with their dams till the end of July. All lambs were raised throughout the day with their dams continuously on a pasture, and at the night in the stable. They suckled their dams and grazed until slaughter. No concentrate was available to ewes or lambs. A total of 60 lambs (30 males and 30 females) of Lika Pramenka breed were randomly chosen to represent their typical breed characteristics.

At the age of five months lambs were transported to a local commercial slaughterhouse and fasted for 12 h with free access to water. All procedures with the lambs were carried out in accordance with the animal welfare rules prescribed by Croatian regulations (16). Before the slaughter, lambs were weighted (slaughter weight). Slaughter and dressing methods followed normal commercial procedures (12). All non-carcass components (stomach with intestines, lungs with heart, liver, spleen, testes and skin with feet) were removed from the body, immediately weighted and recorded. Once the evisceration was conducted, the carcasses were weighted (hot carcass weight) and then chilled at 4 °C for 24 h in a conventional cooler. Linear dimensions were measured after cooling on the intact carcasses (12). For linear dimensions calliper and measuring tape were used. Carcass length was measured from the caudal edge of the last sacral vertebra to the dorso-cranial edge of the atlas. Chest depth was measured as the greatest depth of chest in a horizontal plane of the hanging carcass. Chest width and pelvis width were measured as the greatest width of chest, i.e. pelvis in a horizontal plane of the hanging carcass. Hind leg length was measured from the centre of the tuberosity on the proximal end of the tibia to the distal edge of the tarsus. Following that measurements the carcasses were halved throughout the dorsal midline. The left sides were divided into individual cuts: neck, shoulder, rack, loin, hind leg, shank, belly and flank (Figure 1) (21). Dissection of the individual cuts was carried out obtaining muscle, bone and fat (12). Furthermore, less-priced cuts (head, tail, kidney, kidney knob and channel fat) were removed and weighted from the halved carcasses. Each of them was expressed as a proportion of less-priced cuts and was excluded in the calculations related to carcass composition.

All investigated traits were analysed by MIXED procedure of SAS/STAT software package (20) considering sex and slaughtering season as class fixed effects and slaughter weight as linear regression. Differences of last-square means as well as significance of regression slopes were performed by t-test ($\alpha = 0.05$).

Results and discussion

The results of the slaughter traits, non-carcass composition and linear measurements of Lika Pramenka lambs are presented in Table 1. There was no significant effect of sex on slaughter traits and non-carcass components. The results of slaughter weight, hot carcass weight and dressing percentage are in agreement with those reported for Segureña lambs (18) and for Churra da Terra Quente lambs (19). The results obtained for non-carcass components are partially in accordance

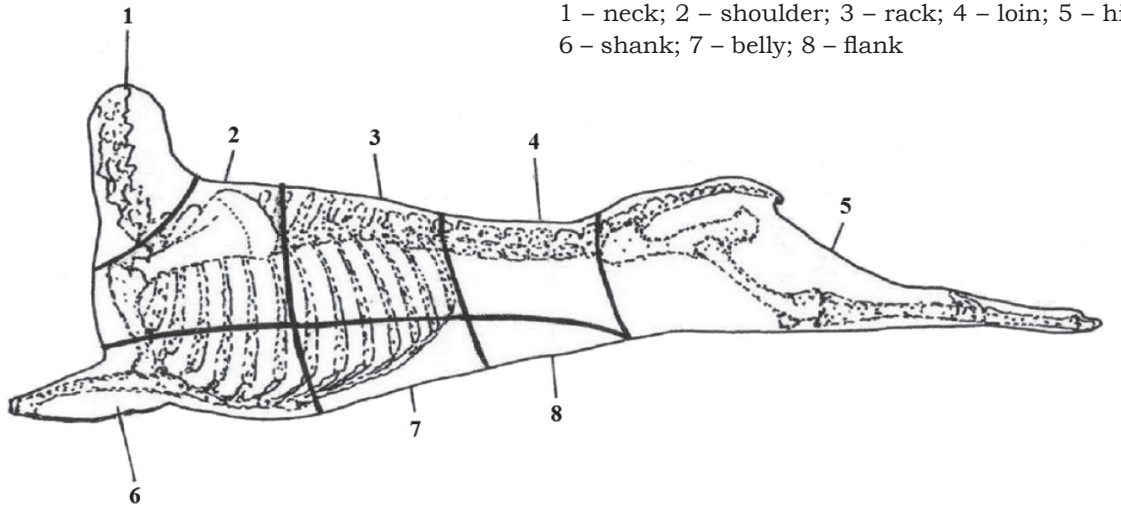

Figure 1: Half lamb carcass with individual cuts (21)

with those reported for improved Jezersko-solčava lambs (24) where male lambs had only significantly higher proportion of liver while difference of any other non-carcass components between male and female lambs was not determined. Since a small increase in lamb slaughter weight may result in higher productivity, and give more flexibility to the production system (19), one of the before mentioned aims was to investigate influence of slaughter weights of Lika Pramenka breed given at the age of 5 months in traditionally rearing system. In the present study increase in slaughter weight from 22.1 to 31.3 kg was followed by increase in hot carcass weight, dressing percentage, stomach with intestines, lungs with hearth and skin with feet ($p \leq 0.01$). Similar results were reported for Awassi lambs (1) where hot carcass weight, dressing percentage and all non-carcass components (except spleen) increased by slaughter weight from 20.9 to 30.5 kg. Slaughtering season significantly affected slaughter weight, hot carcass weight and dressing percentage ($p \leq 0.01$). According to available data from the Croatian Bureau of Statistics, hydro meteorological conditions of the Lika Pramenka breeding area in the present study substantially differed between the slaughtering seasons. These differences in climate conditions indirectly affected food supply via vegetation development which resulted in higher body weight of lambs reared in season 2010. Weight of the stomach with intestines, lungs with heart and liver were also significantly influenced ($p \leq 0.05$) by slaughtering season.

Male carcasses had deeper chests ($p \leq 0.01$) and longer hind leg ($p \leq 0.05$) than female carcasses, while all other measurements were not significantly different. The resulting difference between the overall carcass development in the present study was significantly different from the one reported on the carcasses of Dalmatian Pramenka and Istrian lambs (22). Dalmatian Pramenka and Istrian lambs were reared under semi-extensive production systems and grazed on the natural pastures poorer than those in Lika. The author pointed out that the influence of gender was almost negligible for the most of carcass measurements, and found statistically significant differences only in the width of the chest and pelvis. In Dalmatian Pramenka lambs, females had wider chests while in Istrian lambs males had wider pelvis. Several substantial differences were also reported between carcasses of male and female Segureña lambs (18). Compared to females, males had significantly deeper chest, longer hind leg and wider buttock. Among investigated carcass measurements, it was found that change in slaughter weight significantly affected only chest depth ($p \leq 0.05$) (Table 1). Slaughtering season significantly affected all carcass measurements of Lika Pramenka lambs ($p \leq 0.01$; $p \leq 0.05$). Since the majority of the feeds were supplied from the pasture, it is assumed that the most of these differences in the corpulence between investigated seasons arose due to different forage availability.

Table 1: Slaughter traits, non-carcass components and linear measurements according to sex (S), slaughter weight (SW) and slaughtering season (SS); LSM – least square mean

Trait	Sex (LSM ± SE)		S[1]	b	SW[1]	Season (LSM ± SE)		SS[1]
	Male	Female				2010	2011	
Slaughter weight, kg	27.03 ± 0.12	27.13 ± 0.10	ns	-	-	27.53 ± 0.11	26.62 ± 0.15	**
Hot carcass weight, kg	13.48 ± 0.12	13.54 ± 0.10	ns	0.533	**	13.90 ± 0.11	13.29 ± 0.10	**
Dressing percentage, %	49.87 ± 0.16	49.90 ± 0.12	ns	1.108	**	50.49 ± 0.16	49.92 ± 0.11	**
Stomach with intestines, kg	7.04 ± 0.14	7.07 ± 0.17	ns	0.351	**	7.52 ± 0.17	6.99 ± 0.23	*
Lungs with hearth, kg	0.72 ± 0.01	0.79 ± 0.01	ns	0.019	**	0.85 ± 0.01	0.72 ± 0.01	*
Liver, kg	0.50 ± 0.01	0.57 ± 0.01	ns	-0.008	ns	0.63 ± 0.01	0.43 ± 0.02	*
Spleen, kg	0.13 ± 0.01	0.13 ± 0.01	ns	0.005	ns	0.13 ± 0.01	0.15 ± 0.01	ns
Skin with feet, kg	4.13 ± 0.06	4.15 ± 0.07	ns	0.198	**	4.14 ± 0.07	4.17 ± 0.10	ns
Carcass length, cm	66.26 ± 0.21	66.47 ± 0.23	ns	0.049	ns	66.82 ± 0.23	65.29 ± 0.24	**
Chest width, cm	14.26 ± 0.10	14.16 ± 0.13	ns	0.019	ns	14.32 ± 0.14	13.88 ± 0.17	**
Chest depth, cm	25.77 ± 0.13	24.95 ± 0.16	**	0.110	*	25.67 ± 0.17	25.16 ± 0.20	*
Hind leg length, cm	27.11 ± 0.19	26.45 ± 0.13	*	0.101	ns	27.31 ± 0.29	26.79 ± 0.22	*
Pelvis width, cm	14.45 ± 0.18	14.31 ± 0.10	ns	0.020	ns	14.96 ± 0.11	14.01 ± 0.13	**

[1]Significance level: ns= not significant; *$p \leq 0.05$; **$p \leq 0.01$; b: regression coefficient of slaughter weight

Proportions of individual and less-valuable cuts of Lika Pramenka lambs are presented in Table 2. Male lambs had significantly higher proportion of shoulder ($p \leq 0.05$), hind leg ($p \leq 0.01$), shank ($p \leq 0.05$) and head ($p \leq 0.05$), and significantly lower proportion of rack ($p \leq 0.01$) than females. Higher proportion of neck, shoulder and chuck for males and higher proportion of rack and loin were found for females in improved Jezersko-solčava lambs (24). Higher proportions of neck and shoulder for males were also found in Segureña lambs (18). Differences in other proportions between genders in our and above mentioned studies were found to be non-statistically significant. Among above mentioned studies results vary considerably and could be primarily due to different positions of anatomical cuts or to a different grow rates of each breed.

The change in weight is accompanied by the change of the proportions of individual cuts i.e. the proportion of ribs increases while proportions of the neck, leg and shoulder decrease (14). Nevertheless, for improved Jezersko-solčava lambs was reported that with increased slaughter weight from 29 to 43 kg the percentage of neck, rack and rib with flank increased, and chuck, shoulder and hind leg decreased (24). Additionally, it was reported that increase in slaughter weight of heavier Bafra lambs (30, 35, 40 and 45 kg) was followed by an increase in the proportion of neck, breast + flank and tail, as well as a decrease in the proportion of foreleg and loin (23). In the present study, increase in slaughter weight was followed by a significant increase in proportion of shank ($p \leq 0.05$), belly ($p \leq 0.05$) and less-valuable cuts ($p \leq 0.05$). Among less-valuable cuts proportion of kidney knob ($p \leq 0.05$) and channel fat ($p \leq 0.05$) were also significantly influenced by slaughter weight (Table 2). The remaining individual cuts were not significantly influenced by slaughter weight due to the narrower weight range in the present study. Slaughtering season significantly

Table 2: Proportions of individual and less-valuable cuts according to sex (S), slaughter weight (SW) and slaughtering season (SS); LSM – least square mean

Trait	Sex (LSM ± SE)		S[1]	b	SW[1]	Season (LSM ± SE)		SS[1]
	Male	Female				2010	2011	
Neck	7.72 ± 0.35	7.87 ± 0.41	ns	0.081	ns	7.84 ± 0.39	6.80 ± 0.34	*
Shoulder	14.88 ± 0.28	14.16 ± 0.30	*	-0.172	ns	14.78 ± 0.30	14.26 ± 0.29	ns
Rack	6.85 ± 0.54	8.68 ± 0.56	**	-0.033	ns	7.06 ± 0.57	8.98 ± 0.54	**
Loin	8.55 ± 0.25	8.50 ± 0.26	ns	0.023	ns	8.38 ± 0.26	8.12 ± 0.25	ns
Hind leg	28.22 ± 0.22	27.24 ± 0.23	**	0.112	ns	28.31 ± 0.24	27.19 ± 0.22	*
Shank	14.97 ± 0.36	14.01 ± 0.38	*	0.550	*	13.89 ± 0.34	15.20 ± 0.36	**
Belly	3.90 ± 0.15	4.09 ± 0.15	ns	0.559	*	4.49 ± 0.16	3.42 ± 0.15	**
Flank	2.11 ± 0.13	2.41 ± 0.14	ns	0.025	ns	1.77 ± 0.14	2.81 ± 0.14	**
Less-priced cuts	12.80 ± 0.43	13.04 ± 0.52	ns	0.332	*	13.48 ± 0.35	13.22 ± 0.33	ns
Head [2]	63.51 ± 0.10	62.09 ± 0.11	*	-0.110	ns	62.71 ± 0.25	63.10 ± 0.19	ns
Tail [2]	18.12 ± 0.15	18.19 ± 0.11	ns	0.129	ns	18.11 ± 0.17	18.19 ± 0.16	ns
Kidney [2]	5.50 ± 0.09	5.57 ± 0.10	ns	-0.018	ns	5.41 ± 0.11	5.59 ± 0.10	ns
Kidney knob [2]	7.46 ± 0.18	7.80 ± 0.41	ns	0.433	*	7.43 ± 0.10	7.79 ± 0.10	ns
Channel fat [2]	5.41 ± 0.05	6.35 ± 0.25	ns	0.320	*	6.34 ± 0.05	5.33 ± 0.13	ns

[1]Significance level: ns= not significant; *$p \leq 0.05$; **$p \leq 0.01$; [2]Expressed as a proportion of less-priced cuts; b: regression coefficient of slaughter weight

affected proportion of neck ($p \leq 0.05$), rack ($p \leq 0.01$), hind leg ($p \leq 0.05$), shank ($p \leq 0.01$), belly ($p \leq 0.01$) and flank ($p \leq 0.01$). We assume that the most of these differences arose due to different forage availability and consequently higher or lower physical activity (in search for food).

Proportions of muscle, bone and fat depots of individual cuts and total carcass composition of Lika Pramenka lambs are shown in Table 3. Male lambs had significantly higher proportion of muscle in shoulder ($p \leq 0.05$), rack ($p \leq 0.01$), shank ($p \leq 0.05$), belly ($p \leq 0.001$) and significantly higher bone proportion in loin ($p \leq 0.05$), neck ($p \leq 0.01$) and belly ($p \leq 0.01$) while female lambs had significantly higher fat proportion in all individual cuts (except flank) (Table 3). Lower bone and muscle proportions and higher fat proportions in female lambs indicate that females matured earlier than males. This was also reported in other sheep breeds and at different weight ranges (2, 9, 10). Female Churra da Terra Quente lambs (slaughtered at <8 kg, 8-11 kg and >11 kg) had significantly higher muscle proportion in leg, chump, loin and neck than males (19). On the other hand, no differences were found between gender in intramuscular fat and, except for the shoulder, in bone proportions. Contrary to them, male Manchego lambs (slaughtered at 10, 12 and 14 kg) had higher bone proportion in leg, loin-rib, anterior-rib, shoulder and flank, higher muscle proportion in shoulder and neck, and lower fat proportion in leg, loin-rib, shoulder, flank and neck (9). Nevertheless, it must be considered that it is difficult to compare tissue composition from the individual cuts primarily due to different slaughter weights and dissection methodology. A very accurate method to determine tissue composition of the carcass is the dissection of some individual cuts, such as leg, shoulder and loin (11). Dissection of hind leg and shoulder is more common in the studies due to their high correlation with carcass tissue composition and fact that together constitute over 50% of the lamb carcass (5). According to dissection methodology and slaughter weight, results of our study were comparable with some other reports only in the

Table 3: Proportions of muscle, bone and fat depots of individual cuts and total carcass composition according to sex (S), slaughter weight (SW) and slaughtering season (SS); LSM – least square mean

Trait		Sex (LSM ± SE)		S[1]	b	SW[1]	Season (LSM ± SE)		SS[1]
		Male	Female				2010	2011	
Neck	Muscle	44.19 ± 1.25	44.41 ± 1.59	ns	0.126	ns	44.16 ± 1.61	44.49 ± 1.54	ns
	Fat	21.27 ± 2.15	23.34 ± 2.47	*	0.142	ns	22.74 ± 1.53	22.95 ± 1.47	ns
	Bone	34.54 ± 1.67	32.25 ± 1.92	**	-0.014	ns	33.10 ± 1.19	32.57 ± 1.17	ns
Shoulder	Muscle	50.45 ± 2.42	48.77 ± 2.78	*	0.181	ns	50.48 ± 1.72	50.05 ± 1.65	ns
	Fat	10.76 ± 1.52	12.88 ± 1.74	**	0.127	ns	10.37 ± 1.08	11.41 ± 1.04	*
	Bone	38.79 ± 1.37	38.35 ± 1.57	ns	-0.095	ns	39.15 ± 0.97	38.54 ± 0.96	ns
Rack	Muscle	39.15 ± 2.94	36.66 ± 2.38	**	0.092	ns	37.25 ± 2.10	38.76 ± 2.01	*
	Fat	11.97 ± 1.58	14.81 ± 1.12	**	0.084	ns	14.04 ± 1.55	12.69 ± 1.45	*
	Bone	48.88 ± 1.47	48.53 ± 1.70	ns	-0.014	ns	48.71 ± 1.05	48.55 ± 1.03	ns
Loin	Muscle	38.61 ± 2.23	38.00 ± 2.56	ns	0.072	ns	38.50 ± 1.59	38.10 ± 1.52	ns
	Fat	15.69 ± 1.57	17.83 ± 1.81	**	0.130	ns	16.06 ± 1.12	17.09 ± 1.08	*
	Bone	45.70 ± 1.32	44.17 ± 1.52	*	-0.022	ns	45.44 ± 0.94	44.81 ± 0.93	ns
Hind leg	Muscle	63.57 ± 0.95	63.65 ± 1.09	ns	0.098	ns	63.84 ± 0.67	64.11 ± 0.65	ns
	Fat	7.95 ± 1.04	8.98 ± 1.20	*	0.148	ns	8.93 ± 0.74	7.80 ± 0.71	*
	Bone	28.48 ± 0.70	27.37 ± 0.80	*	-0.089	ns	27.23 ± 0.50	28.09 ± 0.49	ns
Shank	Muscle	45.15 ± 2.44	44.09 ± 2.81	*	0.123	ns	44.24 ± 1.74	44.67 ± 1.67	ns
	Fat	17.07 ± 1.60	18.48 ± 1.84	**	0.315	*	18.38 ± 1.14	18.04 ± 1.10	ns
	Bone	37.78 ± 0.78	37.43 ± 0.90	ns	-0.131	ns	37.38 ± 0.56	37.29 ± 0.55	ns
Belly	Muscle	62.73 ± 2.34	57.62 ± 2.84	***	0.033	ns	60.12 ± 2.29	62.38 ± 2.38	**
	Fat	18.44 ± 2.18	25.82 ± 2.66	***	0.353	*	23.15 ± 2.18	21.56 ± 2.27	**
	Bone	18.83 ± 1.55	16.56 ± 1.78	**	-0.066	ns	16.73 ± 1.08	16.06 ± 1.10	ns
Flank	Muscle	65.48 ± 1.51	65.39 ± 1.22	ns	0.077	ns	65.45 ± 1.72	65.58 ± 1.29	ns
	Fat	34.52 ± 1.68	34.61 ± 1.23	ns	0.080	ns	34.55 ± 1.62	34.42 ± 1.52	ns
Total muscle		59.29 ± 1.04	58.12 ± 1.04	*	0.169	ns	58.60 ± 1.06	59.01 ± 1.05	ns
Total fat		14.49 ± 1.13	16.56 ± 1.15	**	0.262	ns	16.40 ± 1.12	15.46 ± 1.16	*
Total bone		26.22 ± 0.27	25.32 ± 0.38	*	-0.017	ns	25.00 ± 0.39	25.53 ± 0.33	ns

[1]Significance level: ns= not significant; *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$; b: regression coefficient of slaughter weight

hind leg composition. Therefore, in the further text we will discuss about it. Muscle proportion of hind leg in Lika Pramenka lambs was not significantly affected by gender. Contrary to that, male lambs had significantly lower fat proportion ($p \leq 0.05$) and higher bone proportion ($p \leq 0.05$) than females. The difference in muscle proportion of hind leg between male and female improved Jezersko-solčava lambs and their crossbreds with Charollais was also not significant (6). Furthermore, as in the present study, authors reported that female lambs had higher fat proportion and lower bone proportion in the hind leg. Nevertheless, it must be considered that improved Jezersko-solčava lambs and their crossbreds with Charollais, compared to Lika Pramenka lambs, had higher muscle proportion and lower fat and bone proportions in the hind leg. Contrary, male Jezersko-solčava lambs (24) in the hind leg tended to have higher values for muscle and bone proportions, and lower fat proportion than females. Apart from slaughter weight and

dissection methodology these differences may be also due to genetic factors and rearing conditions.

Carcass composition as well as muscle, bone and fat of individual cuts are correlated with carcass weight (8). In accordance with that, numerous researches (1, 10, 18, 23) reported that muscle and bone proportions decreased and fat proportion increased with slaughter weight. In the present study increase in slaughter weight was followed only with fat proportion in shank ($p \leq 0.05$) and belly ($p \leq 0.05$). The non significant differences in carcass composition as well as muscle, bone and fat proportions of individual cuts could be explained because of a narrower range in slaughter weight. Nevertheless, it must be considered that despite of non significant differences, regression coefficients for muscle and fat proportion were positive in all individual cuts while that for bone proportion were negative. Slaughtering season significantly affected ($p \leq 0.05$) fat and bone proportions in the carcass; fat proportion in shoulder, rack, loin, hind leg and belly, and muscle proportion in rack and belly (Table 3). Energy availability, which could be changed throughout different rearing systems and feeds, is highly related with carcass tissue composition (4). Although in the present study rearing system was not changed we assume that different climate conditions within investigated period affected vegetation development and throughout different energy availability influenced the carcass composition.

Conclusions

Results of the present study had evidenced that male and female Lika Pramenka lambs reared under similar conditions and slaughtered at similar age would not have similar expression of all investigated carcass characteristics. Slaughter weight notably affected some of the traits (carcass weight, dressing percentage, fat proportions in shank and belly, kidney knob, channel fat) that could be of interest for buyers. Slaughtering season significantly affected most of the investigated traits. In order to provide more uniform lambs at the market it is necessary to assure typical feeds in unfavourable seasons and slaughter lambs at narrower weight ranges. Typical and "natural" meat products are gaining popularity and have a good acceptance by consumers. Therefore, in the future it is necessary to conduct more detailed studies with sensory evaluation and consumers included.

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VPLIV SPOLA, TELESNE MASE IN SEZONE NA KLAVNE LASTNOSTI JAGNJET PASME LIŠKA PRAMENKA, VZREJENIH V OKVIRU POLEKSTENZIVNEGA PROIZVODNEGA SISTEMA

A. Kaić, B. Mioč, A. Kasap

Povzetek: Namen raziskave je bil oceniti vpliv spola, telesne mase in sezone na lastnosti klavnih trupov jagnjet pasme liška pramenka (30 samcev in 30 samic). Vsa jagnjeta so bila tradicionalno vzrejena v dveh sezonah na naravnih pašnikih in zaklana pri starosti petih mesecev. Moška jagnjeta so imela globlja prsa, daljše zadnje noge, večji delež pleč, zadnjih nog, trupa in glave in manjši delež reber. Jagnjice so imele manjši delež kosti in mišične mase ter večji delež maščobe v posameznih kosih. Klavna teža je znatno vplivala na maso toplega trupa, maso želodca s črevesjem, pljuč s srcem, kože z nogami, na globino prsnega koša ter na delež loja v trebuhu, stegnih in ob ledvicah. Sezona je imela vpliv na vse klavne lastnosti, linearne meritve in netrupne dele (razen vranice in kože z nogami). Poleg tega je sezona vplivala na delež vratu, stegen, zadnjih nog, trebuha, delež kosti in loja, delež maščob v plečih, stegnih, ledjih, zadnjih nogah in trebuhu, ter delež mišic v nogah in trebuhu. Da bi zagotovili enotnejša jagnjeta na trgu, je treba poskrbeti za tipično prehrano v neugodnih sezonah in težo klavnih jagnjet omejiti. Ker so tipični in "naravni" mesni izdelki vedno bolj priljubljeni, je potrebno v študije vključiti tudi senzorično ocenjevanje in potrošnike.

Ključne besede: sestava trupa; linearne meritve; netrupne komponente; klavne lastnosti

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Original Scientific Article

MALLARDS (*Anas platyrhynchos*) - A RISK TO HUMAN HEALTH FROM EXPOSURE TO LEAD SHOTS CONTAMINATING THE ENVIRONMENT

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Summary: The problem around bodies of water used for waterfowl hunting is elevated lead contamination. The aim of the study was to determine which bodily tissues of mallards suffer the most from lead contamination, and whether such contamination can lead to the exceeding of the maximum allowable lead concentrations in meat and giblets set by the EU for poultry. Two groups of hunted mallards were used in the study. One group consisted of ten hunted mallards that spent a part of their life on a pond (experimental group, E). The other group was made up of ten mallards raised without access to a body of water (control group, C). Lead concentrations were determined by high resolution continuum source atomic absorption spectrometry. In experimental group, considerably higher average lead concentrations (mean±SD; mg/kg) were found in breast muscle (E=0.253±0.205; K=0.077±0.031), heart (E=0.272±0.307; K=0.096±0.042), lungs (E=2.721±3.950; K=0.205±0.048), liver (E=7.669±14.048; K=0.287±0.124) and kidneys (E=24.944±30.377; K=0.407±0.106). Significant differences ($P<0.05$) between E and C group were found in breast and heart muscle, as well as in lung and kidney tissue. A comparison between average lead concentrations in experimental group and maximum lead concentration limits in poultry meat and giblets set forth by the EU showed that the maximum concentration limits were statistically significantly exceeded in the case of breast muscle ($P<0.043$) and kidneys ($P<0.032$). It follows from the results that mallards bagged on a pond contaminated with lead from shotgun pellets can pose a risk to human health.

Key words: lead pellets; game; waterfowl; wild duck

Introduction

The problem in the surroundings of bodies of water used for waterfowl hunting is elevated lead contamination (1 - 3). The source of that contamination is the long-term use of lead in the manufacture of shots used in waterfowl hunting (3 – 4). A shotgun cartridge used in waterfowl hunting containing approx. 32 g of lead alloy may be loaded with up to 140 shot pellets (the weight and quantity differing according to the type of cartridge used). Several cartridges may be fired at a single duck, and only a few pellets will remain in the duck's body. The rest of the pellets will be left unnoticed in the environment for many years but they become a possible source of lead contamination of the environment.

In spite of its relatively high stability in the environment (4), lead may pose a serious risk. The risk materializes when lead enters the food chain (5). Waterfowl may mistake lead pellets left lying around bodies of water for feed (seeds) or small pebbles-gastrolites/grit, which birds ingest to

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facilitate food digestion. Ingested pellets will either be soon excreted from the body, or will stay for 18-20 days on average in the proventriculus together with sand and grit (6). Shot pellets retained in the stomach will be fragmented by the action of grit and dissolved by the proventricular acid. Lead salts thus produced are then absorbed into the blood circulation (7). Lead can thus be deposited in various bodily tissues and organs (4). High quantities of ingested lead produce symptoms of acute poisoning, and the birds affected eventually die. Although birds may die of lead poisoning at any time throughout the year, most cases are usually reported at the close of the waterfowl hunting season (8), and in November in particular (9). At lower doses, lead may be deposited for extended periods of time in bodily tissues (10). Under certain circumstances, lead shots may also be the source of environmental contamination. Lead may pass from pellets to sediments at the bottom of bodies of water, and be picked by, and accumulated in, aquatic organisms and plants. Lead from them may enter the organism of waterfowl as part of their diet. The lead ingested as part of contaminated food may be accumulated, with different intensities, in both meat and organs of the birds. Lead-contaminated waterfowl may be a source of lead contamination for bird-hunting predators, scavengers and beast of prey (4, 11). Man can also be exposed to negative effects of lead if he eats lead-contaminated animals. The risk of poisoning is particularly acute in people who are regular game eaters.

One of very common waterfowl raised for hunting in the Czech Republic are mallard ducks. They belong among dabbling ducks, and are often called one of the greatest "gluttons" among waterfowl. Mallard's diet consists mainly of what it finds in water, near the banks and in the close vicinity of bodies of water. Cases of lead poisoning have been known especially amongst waterfowl (5, 7). The mallard, from human nutrition point of view, may be a high-risk lead-contaminated food.

Material and methods

In the study, we used a group of ten mallards raised on a pond in southern Moravia (Czech Republic), which had been used for duck hunting with lead shot for several years. The ten ducks were nagged during the autumn 2010 hunting season (experimental group, E). To rule out lead contamination from lead shot pellets embedded in the body, only ducks killed with steel shot were used. The control group was made up of ten mallard's carcasses coming from the same breeding facility but raised in an enclosure without access to a body of water (control group, C). Samples of the right breast muscle, heart, lung, liver and kidneys were collected from all of the birds. All samples were packed individually in polypropylene bags and frozen (–18°C). Lead concentrations in collected tissue and feedstuff samples were determined by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS) using ContrAA 700 spectrometer (Analytik Jena AG, Germany). Soft tissues were determined by electrothermal atomization. Prior to the determination of lead concentrations, samples were decomposed. Weight of the tissue 1g was fumed with 6 ml nitric acid and 1 ml hydrogen peroxide in a microwave-heated laboratory autoclave (ETHOS SEL, Milestone, Italy). The sample solution was made up to volume 10 ml by water. The detection limit for lead (3σ) was 0.021 mg·kg-1, the reproducibility was expressed from five measurements as RSD 3.7 %. Standard reference materials, DORM-2 (NRC, dogfish muscle), 1577b (NBS, bovine liver) and H-5 (IAEA, animal bone), were used to verify the validity of the method. Statistical analyses were done using Statistica 8.0 for Windows software. After testing for normality, data were subjected to one-way ANOVA and subsequently to Tukey-HSD test or nonparametric ANOVA and Kruskal-Wallis test.

Results

Lead concentrations in breast muscle and individual organs in ducks raised on a pond (experimental group, E) and ducks raised away from the pond (control group, C) are given in Table 1. A comparison of average lead concentrations in breast muscle and individual organs between ducks raised on a pond (experimental group, E) and ducks raised away from the pond (control group, C) is given in Figure 1.

Table 1: Lead concentrations in mallard

	Breast muscle [mg/kg]		Heart [mg/kg]		Lung [mg/kg]		Liver [mg/kg]		Kidneys [mg/kg]	
	K1	E2	K1	E2	K1	E2	K1	E2	K1	E2
min	0.015	0.118	0.015	0.064	0.134	0.129	0.185	0.193	0.247	0.370
max	0.114	0.647	0.158	0.894	0.287	11.77	0.618	35.53	0.595	76.91
SD3	0.031	0.205	0.042	0.307	0.048	3.950	0.124	14.048	0.106	30.377
A4	0.077	0.253	0.096	0.272	0.205	2.721	0.287	7.669	0.407	24.944
P5	0.015		0.090		0.0593		0.114		0.020	

[1] control group raised away from a body of water; [2] a group raised on a pond; [3] standard deviation; [4] arithmetic mean; [5] probability level.

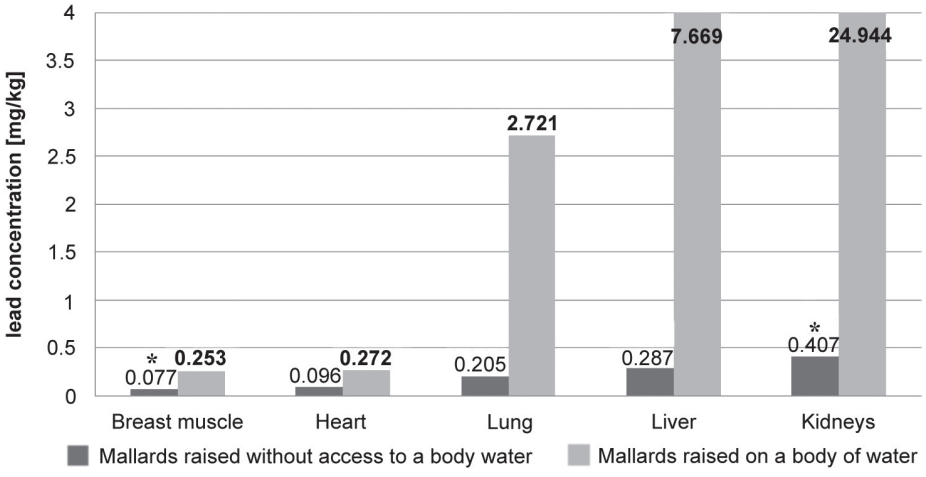

Figure 1: Comparison of lead concentrations between mallards raised without access to a body of water and mallards raised on a body of water

*Statistically significant difference in lead concentrations between mallards raised without access to a body water and mallards raised on a body water.

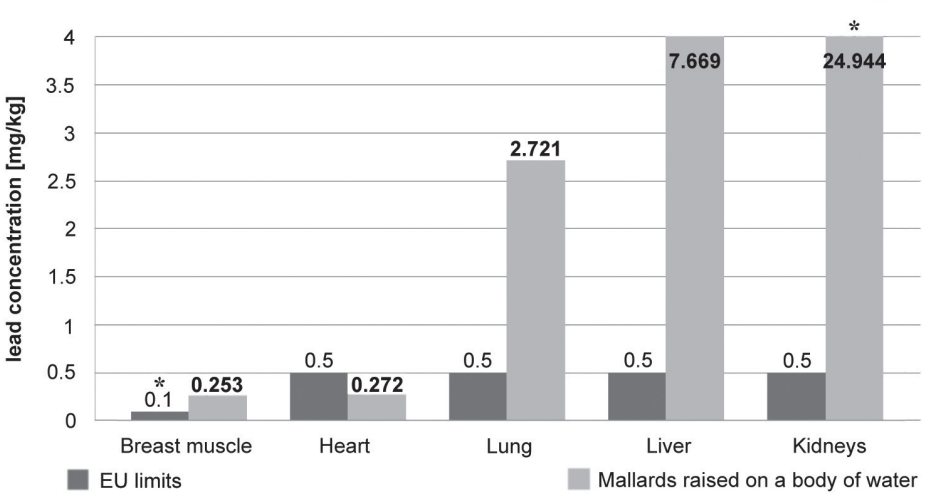

Figure 2: Comparison of lead concentrations in mallards raised on a body of water with EU limits

*Statistically significant difference in lead concentrations between EU limit and in mallards raised on a body of water

From Table 1 and Figure 1 it follows that higher concentration both in the breast muscle, heart, lungs, liver and kidneys were on average found in the group raised on a pond (experimental group) than in the group raised without access to it (control group), and the difference between the experimental and the control groups was statistically significant in the case of breast muscle ($P < 0.015$) and kidneys ($P < 0.020$).

Next, distribution of lead in ducks raised on a pond and exposed to elevated lead contaminations (experimental group) was studied. It follows from Table 1 and Figure 1 that the highest lead concentrations are in the kidneys, followed by the liver, lungs and the heart. The lowest lead concentrations were found in breast muscle. A statistically significant difference was found between mean lead concentrations in the kidneys and lead concentrations in breast muscle ($P < 0.005$), heart ($P < 0.005$) and lungs ($P < 0.016$).

Mean lead concentrations in breast muscle, heart, lungs, liver and kidneys in mallards raised on a body of water contaminated with lead as a result of the site having been used for duck hunting with lead shot for many years (experimental group) were then compared with the maximum allowable limits of lead in meat and giblets set forth by the Commission Regulation (EC) No. 1881/2006 (Figure 2).

From results showed in the Figure 2 it follows that mean lead concentrations found in the mallards raised on a body of water exceed the maximum allowable EU limit set for breast muscle, lungs, liver and kidneys. The amounts in excess of allowable lead limits in foods set forth by the EU were statistically significant in the case of breast muscle ($P < 0.043$) and kidneys ($P < 0.032$). The statistically significant lowest concentrations compared with EU limits were, on the other hand, found in the heart ($P < 0.043$).

Discussion

The environment is contaminated with lead from various sources, with human activities being the most important. Bodies of water used for duck or other waterfowl hunting are among the sites with the highest lead contamination levels. The probability of an elevated lead contamination of bodies of water is the result of the use of cartridges loaded with lead shot in duck or other waterfowl hunting (11). Such pellets left behind around bodies of water after the hunt may be ingested by waterfowl and cause lead accumulation in bodily organs with negative effects on the health of the birds affected, or even causing their death (1, 3 - 4). Under certain circumstances, lead from shot pellets may be released directly to the environment. Lead particles are more readily degradable if the contaminated soil or water is acid in reaction, or if they contain higher concentrations of dissolved oxygen. Lead particles may then be dissolved in soil water, and subsequently assimilated by plants. Contaminated plants may then be the source of lead for the animals that eat those plants (4). In our study, higher lead concentrations in breast muscles and bodily organs were found in ducks raised on a pond (experimental group) compared with ducks raised away from the pond (control group). That confirms the hypothesis that the surroundings of bodies of water where waterfowl hunting takes place belong among environments with elevated lead contamination.

Lead from ingested shot pellets or contaminated food is easily absorbed in the digestive tract. Blood then distributes the lead to body tissues (10). Lead accumulates in vitally important organs (4, 10). The highest lead concentrations are registered several days or even months after exposure in the kidneys and liver. High Pb in bones indicates a long term exposure to lead, and this deposited lead can be mobilized in situations when Ca would be mobilized from bones (12). In our study, we found the highest lead concentrations in the kidneys and liver. That confirms the hypothesis of high lead accumulation in parenchymatous organs, such as liver and kidneys. Our results are in agreement with the previously reported results (13 - 14), where the highest lead concentrations in ducks were found in the liver; high lead concentrations in the kidneys were also mentioned in previously presented studies (4, 10). On the other hand, other authors, who investigated lead concentrations in adult individuals, found the highest levels in bones and breast muscles, kidneys and the brain, and the lowest lead concentrations in the liver (2). The study investigated the use of mallards for the biomonitoring of heavy metal contamination in the environment.

Mallards are game birds whose meat serves as food for people. For that reason it is important to monitor lead contamination in the birds shot from also the human health point of view. Commission

Regulation (EC) No. 1881/2006 (629/2008), which sets maximum limits of certain contaminants in foodstuffs, does not mention any specific maximum allowable limit for lead concentrations in game meat. A comparison of our results with maximum limits for lead in the above regulation set for poultry meat showed that the allowable lead concentrations (0.1 mg/kg) would have been exceeded in eight cases. The maximum allowable limit laid down in the regulation for lead in offal, liver and giblets is 0.5 mg/kg. In our study, lead concentrations in the liver exceeded several times the maximum allowable limit for lead in foodstuffs in six cases. The limit was exceeded in four liver tissue samples, seven pulmonary tissue samples, and heart tissue samples failed to meet the limit in only two cases. From the results reported here it follows that both meat and organs of ducks raised on ponds contaminated with lead from lead shot pellets may pose a health threat to humans once the EU limits for lead contamination have been exceeded.

Cartridges loaded with lead pellets used to be used in the hunting for wild ducks on and around ponds. Lead shot left in and around ponds may be the source of environmental contamination, and a possible source of contamination and even poisoning of waterfowl as well as other animals and even man. Lead can be ingested by waterfowl directly, or lead dissolved in soil water is assimilated by plants and from there, as part of their diet, the lead gets into the organism of waterfowl.

In our study, we looked at the levels of possible lead contamination of the aquatic environment caused by lead shot pellets, and the subsequent impact on the safety of meat and internal organs of waterfowl (mallards). We found that mallards raised on a pond contaminated with shotgun pellets can pose a risk to human health.

Heart is made up of muscle tissue and the results of our study indicate that trends of lead accumulation in it are different from those in parenchymatous organs. It follows from the results that the maximum lead concentration limit for the heart set by the EU should not be the same as that for parenchymatous organs (0.5 mg/kg).

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RACE MLAKARICE (*Anas platyrhynchos*) - TVEGANJE ZA ZDRAVJE LJUDI ZARADI IZPOSTAVLJENOSTI SVINČENIM IZSTRELKOM, KI ONESNAŽUJEJO OKOLJE

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Povzetek: Okrog vodnih teles, ki se uporabljajo za lov vodnih ptic, se pojavlja problem povišane onesnaženosti s svincem. Namen raziskave je bil ugotoviti, katera tkiva mlakaric so najbolj prizadeta zaradi onesnaženja s svincem in ali lahko tako onesnaženje privede do prekoračitev največje dovoljene koncentracije svinca v mesu in drobovini, določene v okviru EU za perutnino. V študiji sta bili uporabljeni dve skupini uplenjenih mlakaric. Ena je bila sestavljena iz desetih ulovljeni mlakaric, ki so del svojega življenja preživele na ribniku (eksperimentalna skupina E), druga pa je bila sestavljena iz desetih mlakaric, ki niso imele dostopa do ribnika (kontrolna skupina C). Koncentracije svinca so bile določene z atomsko absorpcijsko spektrometrijo visoke ločljivosti. V poskusni skupini smo izmerili znatno višje povprečne koncentracije svinca (povprečje ± SD mg / kg) v prsni mišici (E = 0,253 ± 0,205; K = 0,077 ± 0,031), srcu (E = 0,272 ± 0,307; K = 0,096 ± 0,042), pljučih (E = 2,721 ± 3,950; K = 0,205 ± 0,048), jetrih (E = 7,669 ± 14,048; K = 0,287 ± 0,124) in ledvicah (E = 24,944 ± 30,377; K = 0,407 ± 0,106). Razlike ($p < 0,05$) so bile statistično značilne v prsni in srčni mišici, pljučih in tkivu ledvic. Primerjava s povprečnimi koncentracijami svinca v eksperimentalni skupini in mejnih vrednosti koncentracije svinca perutninskega mesa in drobovine, ki jih določa EU, je pokazala, da so bile mejne vrednosti koncentracij statistično značilno presežene v prsni mišici ($P < 0,043$) in ledvicah ($P < 0.032$). Naši rezultati kažejo, da vodni lov mlakaric vodi v onesnaženost ribnikov s svincem in lahko predstavlja tveganje za zdravje ljudi.

Ključne besede: igre; vodne ptice; divja raca

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Original Scientific Article

MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT *Staphylococcus aureus*, ST398 (LA-MRSA), FROM HUMAN SAMPLES

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Summary: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major human pathogen and an important cause of hospital-associated (HA-MRSA) infections. MRSA infections significantly increase morbidity and mortality, affect the increased use of antibiotics and the cost of treatment. During the last decade MRSA has emerged as a significant pathogen also in the community (community-associated; CA-MRSA). In recent years, livestock has been proven to be a source of human infections with the MRSA sequence type (ST) 398 (livestock-associated; LA-MRSA). During the year 2010 all the regional microbiological laboratories took part in the task of monitoring CA-MRSA infections in Slovenia. We included all patients harbouring a MRSA strain that was susceptible to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin or gentamicin. Altogether we collected 151 MRSA isolates of which 15 (9.9%) belonged to a spa type known to be associated with the clone ST398 respectively. Among them 12 isolates belonged to *spa* type t011, 2 isolates to t034 and 1 isolate to t108. We found the staphylococcal cassette chromosome - SCC*mec* type IV or V, and regulatory genes - *agr* type I. None of the isolates were positive for Panton - Valentine leukocidin (PVL), the toxic shock syndrome toxin (tst) and leukocidin *Luk*M. All MRSA isolates were resistant to tetracycline and penicillin. Some of them were also resistant to erythromycin and clindamycin. Most of the LA-MRSA ST398 were isolated from screening specimens of patients from Murska Sobota and Maribor, which are the most important agricultural regions with intensive livestock breeding. Evidence of the presence of LA-MRSA in humans requires a close cooperation of human and veterinary microbiologists. Our goal is to find the epidemiological relation between human and animal hosts, to obtain information on the phenotypic and genotypic characteristics and monitor infections caused by LA - MRSA strains.

Key words: LA-MRSA; human samples; ST398; Slovenia

Introduction

Staphylococcus aureus is present in commensally flora of humans and various animal species, where it can also cause a wide variety of infections (1-5). In humans, *S. aureus* is the most important cause of nosocomial infections, from superficial skin and soft tissue infections to life-threatening infections, while in animals, *S. aureus* mostly causes mastitis in cows and infection in chickens (4, 5).

Methicillin-resistant *S. aureus* (MRSA) was first identified in 1961 in the UK (6). The bacteria managed to spread around the world and became the main cause of hospital-associated (HA-MRSA) infections. HA-MRSA can cause different infections in hospitalized patients of all ages with risk factors, namely MRSA infection or colonization, surgery, admission to a nursing home, use of an indwelling catheter or other medical devices. MRSA infections

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significantly increase morbidity and mortality, affect the increased use of antibiotics and significantly increase the cost of treatment (6). During the last decade MRSA has emerged as a significant pathogen also in the community (community-associated; CA-MRSA) and has caused infections in young and healthy people lacking contact with healthcare (6, 7). In recent years, livestock has been proven to be a source of another kind of MRSA strains in human infections (livestock-associated; LA-MRSA) (1-5). In Europe, LA-MRSA mainly belongs to the sequence type (ST) ST398, while in Asia ST9 is predominant and in Korea ST72 (1, 3, 4, 5). LA-MRSA, HA-MRSA and CA-MRSA differ phenotypically and genotypically (1-6). LA-MRSA ST398 is non-typeable by PFGE using *Sma*I digestion. It carries a smaller staphylococcal cassette chromosome (SCC*mec*) element IV or V, accessory gene regulator (*agr*) type I and lack the major virulence factor of *S. aureus*, such us Pantone-Valentine Leukocidin (PVL), Leukocidin M (*LukM*), toxic shock syndrome toxin 1 (*tst*) and exfoliative toxins (1). LA-MRSA ST398 is generally resistant to tetracycline and in some cases to other antibiotics, such us macrolides, lincosamides, trimethoprim, fluoroquinolones and aminoglycosides (1, 3, 4). The main reservoirs of LA-MRSA ST398 are pigs, poultry or cattle and other animals (1, 2, 3, 5). Humans in close contact with livestock, such as farmers, veterinarians, are often colonized, so therefore the exposure to animals is a risk factor for LA-MRSA carriage (1, 2, 3). In the last years, human infections caused by MRSA ST398 have increasingly been documented in the Netherland, Belgium, Denmark, Germany and Austria, and MRSA ST398 has also been introduced into the healthcare setting mainly in areas with high density of livestock farming (2, 5).

Little is known about the epidemiology of livestock-associated MRSA in Slovenia. Recently, LA-MRSA was found in pigs, pork and dusts (8), but data from humans remains incomplete. The aim of this study was to investigate the presence of LA-MRSA ST398 in human samples, their antimicrobial resistance pattern, toxin gene profile and molecular characterization. For this purpose, we reviewed presumptive CA-MRSA isolates in the strain collection database of the microbiology department of the National Laboratory for Health, Environment and Food based on phenotypic characteristics retrospectively during a 12-month period in the year 2010. Only tetracycline resistant MRSA were included in further analyses.

Material and methods

National collection of presumptive CA-MRSA

Inclusion criteria for presumptive CA-MRSA were based on the antibiotic resistance profile (screening phenotypic pattern). In our national collection, we included only *S. aureus* isolates resistant to cefoxitin and oxacillin, and susceptible to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin or gentamicin (9).

MRSA isolates

In the year 2010, we investigated 151 MRSA isolates with a positive screening phenotypic pattern isolated from asymptomatic carriers or clinical specimens in Slovenian microbiological laboratories. Thirty-one (20.5%) MRSA isolates were tetracycline resistant and were isolated from unrelated patients. All isolates were identified by mass spectrometry (MALDI-TOF MS, Biotyper, Bruker Daltonic GmBH, Bremen, Germany).

Susceptibility testing

The susceptibility testing was performed on Mueller–Hinton agar with the disk diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) (10). The antibiotics tested were penicillin, cefoxitin, vancomycin, gentamicin, tobramycin, kanamycin, erythromycin, clindamycin, tetracycline, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, linezolid, mupirocin and fusidic acid (BD, Maryland). The minimal inhibitory concentration (MIC) for oxacillin and vancomycin was performed using the E-test (bioMerieux, France).

Molecular analysis

All MRSA isolates were molecularly characterized. The SCC*mec* typing was determined by multiplex PCR described previously (11). Methicillin resistance was confirmed by *mec*A and *mec*C PCR (12). Genes encoding accessory gene regulator (*agr*), staphylococcal enterotoxin (*sea* to *see, seg, seh, sei, sek, sel, sen, seo, seu, seq*), toxic shock syndrome toxin-1 (*tst*), staphylococcal exfoliative toxins (*eta, etb, etd*), leukocidin (*luk*M) and Panton-Valentine

leukocidin (*luk*S-*luk*F) were detected by multiplex PCR (13, 14). Amplification, sequencing and analyses of the polymorphic region of the protein A (*spa* typing) and MLST were performed according to a method described previously (15, 16).

Results

Among 31 tetracycline resistant MRSA isolated in the year 2010 from single patients, 15 (48%) isolates belonged to ST398 and presented three different *spa* types: t011 (12 isolates), t034 (2 isolates) and t108 (1 isolate). The SCC*mec* type V was predominant in 14 isolates and the SCC*mec* type IV was found only in one isolate. The *agr* type I and the *mec*A gene were determined in all 15 isolates. 10 isolates with the *spa* type t011 were resistant to penicillin, cefoxitin and tetracycline, 1 isolate with the *spa* type t108 was resistant to penicillin, cefoxitin, tetracycline and chloramphenicol, 2 isolates with the *spa* type's t011 and t034 were resistant to penicillin, cefoxitin, erythromycin, clindamycin and tetracycline. None of the isolates were positive for PVL leukocidin, the toxic shock syndrome toxin and leukocidin *Luk*M. The gene for enterotoxin type O was detected in 6 isolates, the enterotoxin type U was detected in 4 isolates and the enterotoxin type K or I in 1 isolate (table 1). 14 isolates ST398 were isolated from asymptomatic carriers and 1 isolate from a clinical specimen, wound swab. Most isolates were from patients from Murska Sobota and Maribor.

The remaining 16 tetracycline resistant MRSA isolates belonged to 11 different *spa* types: t127 (4 isolates), t015 (3 isolates), t002, t091, t174, t595, t701, t791, t1094, t1218, t11983 (1 isolate of each). The SCC*mec* type IV was predominant in 10 isolates, the SCC*mec* type V was found in 5 isolates and 1 isolate was non-typeable. Five isolates with the *spa* type t015 (3 isolates), t701 and t791 were resistant to penicillin, cefoxitin and tetracycline. One isolate with the *spa* type t791 was PVL positive and belonged to ST72. Isolates with the *spa* type t127 (4 isolates) and t174 (1 isolate) belonged to ST1. These isolates were resistant to penicillin, cefoxitin, erythromycin, kanamycin and tetracycline, while one isolate was also resistant to clindamycin.

Table 1: Characterization of 15 tetracycline resistant MRSA isolated from humans in Slovenia, in the year 2010

Isolate number	Isolate area in Slovenia	Patient gender	Origin	Resistance pattern to antibiotics	PCR		*Spa* type
					SCC*mec* type	Toxin profile	
10-20	MS	F	Screening swab	P	V	-	t011
10-29	MS	F	Screening swab	P, C	V	-	t108
10-39	MS	F	Wound swab	P	V	*selo, selu*	t011
10-40	MS	M	Screening swab	P, E, CC	V	*selo, selu*	t011
10-41	MS	M	Screening swab	P	IV	*selo, selu*	t011
10-46	KP	F	Screening swab	P, E, CC	V	*selo, selu*	t034
10-58	MS	M	Screening swab	P	V	-	t011
10-59	MS	F	Screening swab	P	V	-	t011
10-60	MS	F	Screening swab	P	V	-	t011
10-61	MS	M	Screening swab	P	V	-	t011
10-62	MS	M	Screening swab	P	V	sek	t011
10-67	LJ	F	Screening swab	P, E, CC	V	-	t034
10-115	MB	M	Screening swab	P, E, CC	V	*selo*	t011
10-121	MB	M	Screening swab	P	V	*sei, selo*	t011
10-140	MB	F	Screening swab	P	V	-	t011

Legend:
MS Murska Sobota, KP Koper, LJ Ljubljana, MB Maribor, F female, M male, P penicillin, E erythromycin, C chloramphenicol, CC clindamycin, PCR polymerase chain reaction, SCC*mec* Staphylococcal cassette chromosome *mec*, selo / selu staphylococcal enterotoxin like type O / U, sek / sei staphylococcal enterotoxin type K / I

Discussion

The data about the epidemiology of MRSA among humans in Slovenia are limited. Only few data are available about *spa* types for individual hospitals within a fixed period (9, 17). During the last years, CA-MRSA was found in a low, but increasing number in Slovenian patients. We documented outbreaks of four cases of skin and soft tissue infections due to a CA-MRSA strain obtained from one hospital in 2003 and 2004 (*spa* type t044, ST80) (18). In the year 2005, Mueller-Premru *et al*. reported of PVL-positive CA-MRSA strains among football players (*spa* type t002, ST5 and *spa* type t454, ST152) (19). We confirmed *mec*C-positive MRSA in humans, but no epidemiological connection between humans and animals were found (20). To date, LA-MRSA has not been documented among human isolates. Antimicrobial susceptibility patterns of LA-MRSA are similar to CA-MRSA, and ST398 is one of the predominant clones of CA-MRSA in Europe (21). Therefore, we checked a national collection of presumptive CA-MRSA isolates collected in the year 2010. As we suspected, we found MRSA belonging to a pig-associated clone ST398. MRSA ST398 was mainly detected in colonized patients from rural areas, Murska Sobota and Maribor, where livestock breeding is an important agricultural activity in our country. Human carriage of LA-MRSA is strongly related to direct contact to livestock, but recent studies confirmed the presence of LA-MRSA also in people without risk factors (1, 2, 5). Epidemiological information about animal contact in the patients included in our study was lacking, so the connection between humans and animals could not be confirmed. Despite of that, human colonization upon admission to healthcare indicates strong connection between high pig-farming density and their probably close contact with livestock. Carriage of MRSA ST398 could also be a risk factor in the development of possible infection (1, 2, 4). MRSA ST398 is mainly associated with skin and soft-tissue infections (4, 5), while in our study the MRSA ST398 was detected from wound swab only in one isolate.

The majority of LA-MRSA ST398 lacks many virulence factors that are found in HA-MRSA or CA-MRSA (1, 2, 3, 5). One exception was PVL positive ST398, found in several Chinese isolates (4, 21). In our study, none of the isolates in our study belonging to ST398 carried the leukocidine PVL and *Luk*M or *tst*. The data on the presence of staphylococcal enterotoxin genes in isolates of ST398 are limited. Small number of porcine ST398 isolates carried the enterotoxin genes *seb* or *sek* and *seq* (22). We confirmed different enterotoxins genes (*sek, sei, selo, selu*) in 7 (46.7%) isolates.

In addition to tetracycline resistance, 27% of the MRSA ST398 also presented resistance to macrolides and lincosamides. The resistance to chloramphenicol was detected in one MRSA ST398. Tetracycline resistance could be a good phenotypic marker for the detection of potential LA-MRSA, but we should be aware that different HA-MRSA and CA-MRSA clones are circulating in Slovenia and some of them are also resistant to tetracycline (17-22). Other STs (*spa* type - number of isolate) detected in this study, which genetically are not associated to LA-MRSA ST398, were ST1 (t127-4, t174-1), ST5 (t002, t1094, t11983-1), ST6 (t701-1), ST7 (t091-1), ST22 (t1218-1), ST45 (t015-3), ST72 (t791-1) and ST152/377 (t585-1). Most of these isolates were also resistant to kanamycin and other antimicrobial agents.

Evidence of the presence of LA-MRSA in humans requires a close cooperation of human and veterinary microbiologists. In the future, we all want to find the epidemiological relation between both hosts, to acquire information on the phenotypic and genotypic characteristics and to monitor infections caused by LA - MRSA strains.

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MOLEKULARNA OPREDELITEV PROTI METICILINU ODPORNE BAKTERIJE *Staphylococcus aureus*, KI PRIPADA KLONU ST398 (LA-MRSA) IZ HUMANIH VZORCEV

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Povzetek: Proti meticilinu odporna bakterija *Staphylococcus aureus* (MRSA) ima v zdravstvu zelo velik pomen. Okužbe s to bakterijo pomembno povečajo obolevnost, umrljivost, vplivajo na povečano rabo antibiotikov in pomembno povečujejo stroške zdravljenja. MRSA povzroča okužbe v bolnišničnem (angl. *hospital-acquired*; HA-MRSA) in v domačem okolju (angl. *community-acquired*; CA-MRSA), v zadnjih letih pa novo grožnjo za okužbe pri ljudeh predstavljajo rejne živali. Te so z LA-MRSA (angl. livestock-associated) večinoma kolonizirane, obravnavane bakterije pa pri njih le redko povzročajo okužbe. LA-MRSA najpogosteje povezujejo s sekvenčnim tipom (ST) ST398 oz. klonskim kompleksom (CC) CC398.
Od 1.1.2010 do 31.12.2010 smo v mikrobioloških laboratorijih po Sloveniji, ki opravljajo mikrobiološko diagnostiko, zbirali seve CA-MRSA. V raziskavo smo vključili le izolate, ki so bili odporni proti oksacilinu oz. cefoksitinu in občutljivi vsaj za dva antibiotika, in sicer za ciprofloksacin, eritromicin, klindamicin ali gentamicin. Po tem kriteriju smo zbrali 151 izolatov MRSA, od katerih jih je 15 (9,9 %) pripadalo tipom *spa*, ki jih povezujejo s klonom ST398 oz. MRSA rejnih živali. V sekvenčni tip ST398 smo uvrstili 12 izolatov s tipom *spa* t011, 2 izolata s tipom *spa* t034 in 1 izolat s tipom *spa* t108. Dokazali smo stafilokokni kromosom kasete - SCC*mec* tip IV ali V in regulatorni tip genov - *agr* I. Noben izolat ni imel zapisov za levkocidin Panton-Valentine (PVL), toksin toksičnega šok sindroma (tst) in levkocidin *Luk*M. Vsi izolati MRSA so bili odporni proti tetraciklinu in penicilinu, nekateri tudi proti eritromicinu in klindamicinu. Večino LA-MRSA s sekvenčnim tipom ST398 smo dokazali iz nadzornih kužnin pri ljudeh s področja Murske Sobote in Maribora, kar nakazuje kolonizacijo oz. nosilstvo predvsem pri prebivalcih, ki živijo na območju, kjer sta razvita poljedelstvo in živinoreja.
Dokaz prisotnosti LA-MRSA tudi pri ljudeh predstavlja medicinskim in veterinarskim mikrobiologom velik izziv za medsebojno sodelovanje, katerega cilj je ugotoviti epidemiološko povezavo med obema gostiteljema, pridobiti informacije o fenotipskih in genotipskih lastnostih teh sevov LA-MRSA ter slediti pogostosti okužb, povzročenih s sevi LA-MRSA.

Ključne besede: LA-MRSA; humani vzorci; ST398; Slovenija

Namen ustanovitve in delovanja podjetja MD svetovanje d.o.o. je pomagati podjetjem pri poslovanju z nudenjem produktov in storitev, ki ne spadajo v osnovno dejavnost podjetja. To dosegamo s celovito ponudbo predstavljenih produktov in storitev.

Zato smo naš moto Skupaj bomo uspešnejši! nadgradili še z motom in sloganom Vse za Vas na enem mestu!

Vizija

Postati vodilna neodvisna družba s celotno ponudbo za podjetja in posameznike na enem mestu in na ta način prihraniti podjetjem in posameznikom čas in denar.

Vse to nam bo uspelo s trdim delom in kakovostno izvedbo storitev in zaupanih nam nalog, predvsem če bomo sledili naslednjim načelom:

- zagotavljanje celovite ponudbe,
- vedno delo v dobro stranke,
- strokoven razvoj,
- organizacijsko izpopolnjevanje,
- zagotavljanje visoke stopnje kakovosti storitev z upoštevanjem predlogov naših strank,
- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
- kakovost izvajanja storitev,
- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
- mestu zagotovi vse dejavnosti, ki ne spadajo v njegovo osnovno dejavnost.

Prednosti poslovanja z nami:

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