UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

Polish Journal of Natural Sciences

29

(2/2014)

PUBLISHER UWM
OLSZTYN 2014

The Polish Journal of Natural Sciences is indexed and abstracted
in Biological Abstracts and Biosis Previews

The print edition is the primary version of the Journal

The Journal is also available in electronic form on the websites
http://www.uwm.edu.pl/polish-journal/ (home page)
http://wydawnictwo.uwm.edu.pl (subpage *Czytelnia*)

PL ISSN 1643-9953

PUBLISHER UWM OLSZTYN

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Ark. wyd. 7,3, ark. druk. 6,0, nakład 110 egz.
Druk – Zakład Poligraficzny UWM w Olsztynie
zam. nr 456

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 105–117, Y. 2014

THE EFFECT OF DIETARY PROPOLIS SUPPLEMENTATION ON THE GROWTH PERFORMANCE OF BROILER CHICKENS

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K e y w o r d s: broiler chicken, propolis, antibiotic growth promoter.

A b s t r a c t

The experimental materials comprised 400 Ross 308 chickens (200 ♂ and 200 ♀), divided randomly into four groups kept in 16 pens, as follows: a negative control group (I) – a diet without additives, a positive control group (II) – a diet supplemented with a combination of the antibiotic growth promoter flavomycin (10 mg kg^{-1} feed) and the coccidiostat robenidine (500 mg kg^{-1} starter and grower feed), and two experimental groups – diets supplemented with chemically standardized propolis – 10 mg kg^{-1} feed in group 3 and 50 mg kg^{-1} feed in group IV. The body weights of chickens were determined once a week. Feed intake, mortality and culling rates were monitored regularly. At the end of the experiment, on day 42, 12 chickens (6 ♂ and 6 ♀) were selected from each group for slaughter and carcass quality analysis. The final body weights of chickens, feed intake (kg) per kg body weight and carcass weight during the rearing period did not differ significantly between groups. The lowest feed intake per kg lean meat was noted in chickens fed a diet with 50 mg propolis/kg feed (3.457 kg), and the highest – in broilers fed a diet with 10 mg propolis/kg feed (3.611 kg). The weight of carcass and selected carcass parts (neck, breast, legs) tended to increase in chickens that received propolis, although the noted differences were statistically non-significant. The percentage content of legs in the carcass was significantly lower in chickens fed a diet with an antibiotic growth promoter (AGP). The legs of chickens fed 50 mg propolis had a significantly higher muscle content and a lower fat content, compared with the legs of birds fed a diet with AGP.

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WPŁYW DODATKU PROPOLISU DO PASZY NA WYNIKI ODCHOWU KURCZĄT BROJLERÓW

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S ł o w a k l u c z o w e: kurczęta brojlery, propolis, antybiotykowy stymulator wzrostu.

A b s t r a k t

Materiał do badań stanowiło 400 piskląt Ross 308 (200 ♂ i 200 ♀). Ptaki podzielono losowo w 16 kojcach na 4 grupy: kontrolną negatywną (I) – pasza bez dodatków, kontrolną pozytywną (II) – pasza z dodatkiem flawomycyny w ilości 10 mg kg^{-1} paszy i kokcydiostatyku (robenidyna w ilości 500 mg kg^{-1} paszy starter i grower) oraz dwie grupy doświadczalne – pasza tylko z dodatkiem standaryzowanego chemicznie propolisu: grupa III – 10 mg kg^{-1} paszy, grupa IV – 50 mg kg^{-1} paszy. Raz w tygodniu kontrolowano masę ciała kurcząt oraz na bieżąco zużycie paszy, upadki i brakowania. Na koniec odchowu, czyli w 42 dniu, pobrano do uboju i oceny rzeźnej po 12 kurcząt (6 ♂ i 6 ♀) z każdej grupy. Kurczęta z poszczególnych grup nie różniły się na koniec odchowu istotnie pod względem masy ciała oraz zużycia paszy (kg) na 1 kg masy ciała i tuszki. Na wyprodukowanie 1 kg mięsa najmniej paszy zużyły kurczęta żywione paszą z dodatkiem propolisu w ilości 50 mg kg^{-1} – 3,457 kg, a najwięcej ptaki otrzymujące paszę z dodatkiem propolisu w ilości 10 mg kg^{-1} – 3,611 kg. Pomimo braku różnic potwierdzonych statystycznie zaznaczyła się tendencja w kierunku większej masy tuszki oraz niektórych jej elementów (szyi, części piersiowej i nóg) u kurcząt otrzymujących w dawce propolis. Istotnie mniejszy udział nóg w tuszce zanotowano u ptaków z grupy, w której zastosowano w paszy antybiotykowy stymulator wzrostu (ASW). Procentowa zawartość pozostałych wyrębów w masie tuszki była podobna u ptaków ze wszystkich grup. Nie stwierdzono wpływu sposobu żywienia na masę mięsa, tłuszczu ze skórą, kości oraz podrobów. Jedynie umięśnienie nóg kurcząt żywionych paszą z dodatkiem 50 mg propolisu było statystycznie istotnie większe, a otłuszczenie mniejsze w porównaniu z ptakami otrzymującymi ASW.

Introduction

Propolis, collected by honey bees from botanical sources, is used as a sealant in the hive. It is a potent anitibacterial, antiviral and antifungal agent (OTA et al. 2001, SANTOS et al. 2002, SAWAYA et al. 2002) with antioxidant (CASTALDO and CAPASSO 2002, BURDOCK 1998, NAGAI et al. 2003), liver-protecting (BANSKOTA et al. 2001), anti-inflammatory (WANG et al. 1993) and anti-cancer properties (BANSKOTA et al. 2002). Due to its highly variable chemical composition, propolis does not support the development of antibiotic resistance among pathogenic bacteria (KĘDZIA and HOŁDERNA-KĘDZIA 1996). According to some authors (BIAVATTI et al. 2003, DENLI et al. 2005), propolis may be an effective natural alternative to antibiotic growth promoters in poultry. This is an important consideration, since the use of antimicrobial

growth promoters (AGPs) in animal feed was banned in the European Union on 1 January 2006.

Propolis stimulates the body's immune system and thus it may improve the growth performance and health status of chickens and laying hens (by reducing mortality rates and improving feed conversion), carcass value and meat quality characteristics as well as the welfare of birds (BIAVATTI et al. 2003, BONOMI et al. 2002, LETIN et al. 2010, DENLI et al. 2005, LI and ZHANG 2002, ROODSARI et al. 2004, ZENG et al. 2004). Propolis has been shown to stimulate lymphocyte proliferation and antibody production after immunization. Depending on the dose and time of administration, propolis components may enhance T lymphocyte conversion. As demonstrated by CHEN et al. (1999) and HU et al. (1998), propolis extracts boost immune system development and stimulate the activity of T and B lymphocytes in broiler chickens. In a study by ROODSARI et al. (2004), chickens fed 250 mg propolis per kg feed were characterized by significantly higher body weights and lower feed intake per kg body weight gain, compared with birds that received diets without propolis supplements. In an experiment performed by ZENG et al. (2004), a combination of flower pollen and propolis at a ratio of 2.5:1, used as a feed additive, increased the body weights of chickens by nearly 10% in comparison with the control group. LI and ZHANG (2002) reported that supplementing diets for broiler chickens with 2.5% propolis contributed to higher weight gains and higher feed efficiency, thus increasing production profitability by almost 10%. DENLI et al. (2005) reported that the addition of 0.5 to 1.5 g propolis per kg feed resulted in a significant increase in the body weights of quails, higher feed efficiency and an increase in the serum levels of HDL cholesterol. BONOMI et al. (2002) demonstrated a beneficial influence of propolis on the growth performance of ducks (including higher body weights and daily gains, and higher feed efficiency), carcass dressing percentage, carcass lean content, meat tenderness and digestibility. An increase in the carcass dressing percentage of broiler chickens due to dietary propolis supplementation was also observed by KLECZEK et al. (2007).

Previous research into the use of propolis as a substitute for AGPs has yielded promising results. Therefore, the aim of this study was to determine the effect of diet supplementation with standardized propolis on the growth performance and carcass value of broiler chickens.

Material and Methods

The experimental materials comprised 400 Ross 308 chickens, divided into four groups of males and females, with two replicates of 25 birds per pen

(200 ♂ and 200 ♀ in total). All birds were kept under identical housing conditions. The birds were fed *ad libitum* starter, grower and finisher diets, from day 1 to 14, from day 15 to 28 and from day 29 to 42, respectively (Table 1). Chickens transported from the hatchery were assigned to two control and two experimental groups, as follows: a negative control group (I) – a diet without additives, a positive control group (II) – a diet supplemented with a combination of the antibiotic growth promoter flavomycin at 10 mg kg^{-1} feed and the coccidiostat robenidine at 500 mg kg^{-1} starter and grower feed, and two experimental groups – diets supplemented with chemically standardized propolis (imported from Brazil) at 10 mg kg^{-1} feed in group III and 50 mg kg^{-1} feed in group IV. Feed composition, as provided by the manufacturer, in given in Table 1.

Table 1

Nutritive value of diets

Nutrients	Diet		
	Starter	Grower	Starter
Energy [kcal]	13.0	12.7	12.6
Total protein [%]	22.50	18.70	17.90
Lysine [%]	1.30	0.99	0.96
Methionine+cystine [%]	0.95	0.83	0.74
Threonine [%]	0.93	0.74	0.70
Calcium [%]	1.05	1.17	0.96
Available phosphorus [%]	0.55	0.55	0.56
Sodium [%]	0.16	0.14	0.14
Vitamin A IU	20206	15299	10356
Vitamin D_3 IU	4000	3000	2000
Vitamin E mg	71.5	57.7	43.1
Crude fiber [%]	3.23	3.83	4.15

Due to its heterogeneous chemical composition, propolis used as a dietary supplement undergoes microbiological and/or chemical standardization. The propolis used in this experiment was chemically standardized to determine its concentrations of flavonoids expressed as galangin. The biological activity and medicinal properties of propolis are largely determined by galangin and pinocembrin content (KĘDZIA and HOŁDERNA-KĘDZIA 1996). Propolis dry extract standardized to contain 12% galangin, in powder form, was mixed with dry feed. Diet supplementation with propolis at 50 mg kg^{-1} did not significantly increase feed cost per chicken.

The body weights of chickens were determined once a week, and feed intake, mortality and culling rates were monitored regularly. At the end of the experiment, on day 42, 12 chickens (6 ♂ and 6 ♀) were selected from each

group for slaughter and carcass quality analysis, by stratified sampling (the birds were arranged in ascending order). Following 12-hour feed restriction, the birds were weighed, sacrificed by cervical dislocation, bled, plucked and eviscerated. The gastrointestinal tract, lungs, trachea, heart, abdominal fat and crop were removed. The heads and feet were cut off (between the occipital condyle and the atlas, and at the carpal joint, respectively). Carcasses were chilled at +4°C for 12 hours, they were weighed and divided into the following parts:

1. Neck – along the line connecting the cephalad borders of the coracoids.
2. Wings – at the shoulder joints.
3. Legs – at the hip joints (from the process of the pubis through the groin towards the back, along the vertebral column, starting from the anterior border of the pelvis), the thigh was separated from the drumstick by cutting through the stifle joint.
4. Breast portion – cutting through the cartilaginous adhesions of the ribs, from the lower border of the sternum to the coracoids.
5. Back and loin – the remaining part of the carcass.

The above carcass parts were weighed and dissected into lean meat, bones, skin (including subcutaneous fat), and intermuscular fat. The superficial and deep breast muscles (*Pectoralis major*, *Pectoralis minor*) were separated by cutting along the sternal crest, clavicle and coracoids, and along the line of the attachment of these muscles to the ribs. Tissue components were weighed accurate to 0.1 g. In this study, the term lean meat weight refers to the weight of muscle tissue without intermuscular fat which was separated during carcass dissection. The weight of fat and skin comprises abdominal fat (surrounding the abdominal organs), intermuscular fat and skin including a subcutaneous fat layer which are difficult to separate in poultry. The weight of bones is the weight of all osseous elements in the carcass separated by dissection.

The European Broiler Index (EBI) and the European Production Efficiency Factor (EPEF) were calculated as follows:

$$\text{EBI} = \frac{A^2 \cdot 10\,000}{B \cdot C \cdot D}$$

where:
A – final body weights of broilers [kg];
B – number of fattening days;
C – initial number of chickens;
D – total feed intake [kg].

$$\text{EPEF} = \frac{E \cdot F}{G \cdot H} \cdot 100\%$$

where:
E – average final body weights of broilers [kg];
F – survival rates [%];
G – feed intake [kg] per kg body weight gain;
H – duration of the rearing period (days).

EBI and EPEF are calculated to assess efficiency in broiler production. Differences in the calculated values of the indices are due to the fact that EPEF relies on the survival rates of chickens and feed intake per kg body weight gain, and EBI – on the initial number of chickens, their final body weights and total feed intake.

Carcass value was calculated as the ratio between carcass weight including giblets and the live weight of chickens.

Statistical analysis included:

– statistical characteristics of the analyzed traits – arithmetic means and coefficients of variation (CV);

– the significance of differences between means in dietary treatments and sex groups (two-way ANOVA, dietary treatments x sex, i.e. 4 x 2, F test F, StatSoft, 2008). Data presented in Table 2 (feed intake, mortality/survival rates, EBI and EPEF) were analyzed by ANOVA with two elements per subgroup (mean values per pen) (RUSZCZYC 1978).

Table 2
Feed intake and mortality/survival rates of chickens, the European Broiler Index [EBI] and the European Production Efficiency Factor (EPEF)

Specification	Group				Gender	
	I	II	III	IV	♂	♀
Feed intake [kg] per kg:						
body weight	1.755	1.741	1.778	1.743	1.720	1.789**
carcass weight	2.429	2.378	2.441	2.380	2.367	2.447**
lean meat weight	3.564^{ABab}	3.530^{ABbc}	3.611^{Aa}	3.457^{Bc}	3.463	3.618**
Mortality rates: number of birds	2	3	2	3	6	4
[%]	2.00	2.88	1.96	3.00	2.96	1.96
Survival rates [%]	98.00	97.12	98.04	97.00	97.04	98.04
EBI	358^{ABab}	369^{Aa}	344^{Bb}	348^{ABb}	386**	323
EPEF	347	355	348	346	378**	318

Means followed by capital letters or ** are significantly different at $\alpha = 0.01$, means followed by small letters or * are significantly different at $\alpha = 0.05$

Results and Discussion

The body weights of day-old broilers were comparable (approximately 43 g) in all groups (Table 3). At 1 week of age, chickens fed propolis-supplemented diets (groups III and IV) were significantly lighter than control group (I) birds fed a diet without propolis and AGP. From week 2 to the end of the experiment, the body weights of chickens did not differ significantly (Table 3). A significant difference in the body weights of males and females was observed from week 3 (887.4 g ♂ and 859.4 g ♀) until the end of the experiment (2921.1 g and 2479.8 g respectively, Table 3). A statistical analysis revealed no feeding x sex interaction for body weight and other traits of chickens, discussed later. Our results corroborate the findings of ROODSARI et al. (2004), and SHALMANY and SHIVAZAD (2006), who demonstrated that the body weights of chickens fed a diet supplemented with 50 mg propolis per kg of feed were comparable with the body weights of chickens fed a diet without the supplement, whereas the body weights of birds fed 250 mg propolis increased significantly, compared with the control group. ZENG et al. (2004) also noted an increase (by almost 10%) in the body weights of broilers given a combination of flower pollen and propolis (at a 2.5:1 ratio), in comparison with the control group. Quails that received propolis at 0.5 to 1.5 g kg^{-1} feed had significantly higher body weights than those fed a non-supplemented diet, and similar to the body weights of birds given an antibiotic-supplemented diet (DENLI et al. 2005).

Table 3

Body weights of chickens in successive weeks of the study [g]

Age [weeks]	Statistics	Group				Gender	
		I	II	III	IV	♂	♀
0	mean	43.0	43.2	43.6	42.7	42.6	42.9
	CV	6.71	8.51	6.31	6.94	7.33	7.23
1	mean	160.0^{A}	156.8^{AB}	153.1^{B}	151.4^{B}	154.1	156.6
	CV	9.81	10.89	10.80	9.69	10.15	10.80
2	mean	436.7	424.3	431.0	424.7	432.0	426.4
	CV	10.52	11.81	12.23	11.08	10.97	11.92
3	mean	870.3	855.9	876.5	892.1	887.4**	859.4
	CV	11.78	12.27	12.52	10.54	11.68	11.84
4	mean	1474.8	1470.5	1460.0	1508.4	1539.5**	1417.6
	CV	12.28	11.47	12.46	12.11	10.63	12.16
5	mean	2112.9	2149.8	2178.1	2168.2	2294.5**	2015.4
	CV	15.83	10.47	13.10	12.04	12.09	9.98
6	mean	2674.0	2730.0	2682.4	2712.9	2921.1**	2479.8
	CV	16.09	11.36	13.07	13.16	11.71	9.03

Means followed by capital letters or ** are significantly different at $\alpha = 0.01$

Feed intake [kg] per kg body weight and carcass weight was similar in all groups (Table 2). The lowest feed intake per kg lean meat was noted in chickens fed a diet with 50 mg propolis/kg feed (group IV – 3.457 kg), and the highest – in broilers fed a diet with 10 mg propolis/kg feed (group III – 3.611 kg). Feed intake per kg body weight, carcass weight and lean meat weight was significantly higher in females than in males (Table 2). In a study by ROODSARI et al. (2004), chickens fed 250 mg propolis per kg feed were characterized by significantly lower feed intake per kg body weight gain than birds that received no propolis supplements, whereas lower propolis doses had no effect on feed conversion. Similar results were reported by SHALMANY and SHIVAZAD (2006) who found that only higher propolis doses (200–250 mg kg^{-1} feed) improved feed efficiency. High dietary inclusion levels of propolis (0.5–1.5 g kg^{-1} feed) contributed to better feed conversion in Japanese quails, compared with birds fed a control diet and a flavomycin-supplemented diet (DENLI et al. 2005).

Mortality [number of birds and %] and survival [%] rates were similar in all dietary treatments, and in males and females (Table 2). An absence of significant differences in mortality rates, culling rates and feed conversion between groups could result from too low inclusion levels of propolis (10 and 50 mg kg^{-1}), which corroborates the research findings cited above.

Production efficiency was assessed using EBI and EPEF. EBI values were insignificantly higher than EPEF values (Table 2). EBI reached the highest level of 369 points in control group II (a diet with AGP), and the lowest values of this index were noted in experimental groups fed propolis-supplemented diets (group III – 344 points, group IV – 348 points). Similar EBI values in broiler chickens fed *ad libitum* and subjected to quantitative feed restriction were reported by Wawro et al. (2004). EPEF also reached the highest level (355 points) in group II, but the values of this index noted in the other groups were only 7–9 points lower (Table 2). The above findings are only partially consistent with those of LI and ZHANG (2002) who reported that supplementing diets for broiler chickens with 2.5% propolis contributed to higher weight gains and higher feed efficiency, thus increasing production effectiveness by 9.7%, in comparison with birds that received no dietary propolis supplementation.

Table 4 presents carcass weight [g] and the weight and percentage content of primal cuts in the carcass. The weight of carcass and selected carcass parts (neck, breast, legs) tended to increase in chickens fed propolis. DENLI et al. (2005) observed a considerable increase in carcass weight in quails fed a propolis-supplemented diet (1–1.5 g kg^{-1} feed), compared with those fed a diet containing no additives. The percentage content of legs in the carcass was higher in broilers fed a diet without supplements and propolis-supplemented diets (33.49%, 32.50% and 33.04% in groups 1, 3 and 4, respectively), and significantly lower in chickens fed a diet with AGP (32.14%). The proportions of the other primal cuts in total carcass weight were comparable in all groups.

Table 4

Weight [g] and content [%] of primal cuts in the carcasses of chickens aged 6 weeks

Specification	Statistics	Group				Gender	
		I	II	III	IV	♂	♀
Weight [g] of:							
carcass	mean	1910.8	1891.1	1952.5	1937.0	2097.0**	1749.5
	CV	14.34	8.52	16.03	12.49	9.56	9.07
neck	mean	68.1	65.0	71.5	73.4	76.4**	62.05
	CV	21.41	19.01	17.27	16.11	17.07	9.61
breast	mean	713.0	736.2	750.7	734.8	787.7**	679.7
	CV	14.34	8.97	16.22	13.68	10.57	11.72
back	mean	306.1	306.2	314.5	305.2	334.5**	281.6
	CV	14.05	11.60	14.92	9.68	8.59	10.31
legs	mean	642.0	608.1	639.0	642.1	704.7**	559.4
	CV	17.19	10.54	18.14	15.75	11.32	9.00
wings	mean	177.3	175.4	177.0	180.9	192.0**	163.3
	CV	12.65	10.61	13.77	11.19	8.70	9.12
Content [%] in total carcass weight:							
neck	mean	3.58	3.45	3.68	3.81	3.65	3.61
	CV	19.35	17.62	12.05	13.41	13.52	14.58
breast	mean	37.37	38.95	38.42	37.90	37.55	38.80*
	CV	5.66	4.75	3.68	4.04	4.15	4.44
back	mean	16.04	16.16	16.10	15.84	15.99	16.10
	CV	5.09	4.71	3.77	6.47	4.21	5.34
legs	mean	33.49^{a}	32.14^{b}	32.50^{ab}	33.04^{ab}	33.59**	31.99
	CV	4.49	5.27	3.00	4.23	4.38	3.19
wings	mean	9.31	9.29	9.11	9.37	9.19	9.35
	CV	5.56	9.21	4.64	6.49	7.41	5.66

Means followed by capital letters or ** are significantly different at $\alpha = 0.01$, means followed by small letters or * are significantly different at $\alpha = 0.05$

The differences in the body weights of males and females led to significant differences in the weight of carcass and carcass parts, which were higher in males than in females (Table 4). Females, compared with males, were characterized by higher breast weight and lower leg weight (38.80% vs. 37.55% and 31.99% vs. 33.59%, respectively).

Carcass dissection showed that dietary treatments had no effect on the weight of lean meat, skin with fat, bones and giblets (Table 5). However, chickens fed propolis-supplemented diets tended to have higher weight of lean meat, bones and giblets and lower weight of skin with fat. Such a trend was also noted with respect to the percentage content of the analyzed carcass parts in total carcass weight (Table 5). DENLI et al. (2005) demonstrated that dietary propolis supplementation had no effect on the weight of abdominal fat and giblets in quails.

Table 5

Carcass composition [g, %] in chickens aged 6 weeks

Specification	Statistics	Group				Gender	
		I	II	III	IV	♂	♀
Weight [g] of:							
lean meat	mean	1302.6	1274.5	1323.9	1334.6	1434.5**	1183.3
	CV	14.90	8.99	16.48	13.10	9.71	8.91
skin with fat	mean	299.3	317.0	317.7	294.0	319.6	294.4
	CV	17.04	14.22	20.87	15.77	14.73	19.56
bones	mean	239.1	232.9	244.7	246.7	271.1**	210.6
	CV	16.52	11.82	17.66	14.30	8.43	7.42
giblets	mean	75.6	76.0	77.5	78.3	84.8**	68.87
	CV	18.80	14.07	13.10	13.73	7.93	11.19
Content [%] in total carcass weight:							
lean meat	mean	68.15	67.40	67.79	68.85	68.42	67.67
	CV	1.99	2.88	2.97	1.83	2.20	2.70
skin with fat	mean	15.67	16.79	16.25	15.18	15.21	16.73**
	CV	12.14	12.68	15.40	9.74	8.07	14.58
bones	mean	12.52	12.30	12.54	12.75	12.97**	12.08
	CV	8.80	6.43	6.38	7.14	5.45	5.63
giblets	mean	3.95	4.02	4.00	4.05	4.06	3.95
	CV	10.94	11.37	11.35	9.67	9.02	9.56
Carcass dressing percentage	mean	76.21	77.17	76.46	77.39	76.47	77.14
	CV	2.93	1.47	2.18	1.01	1.59	2.41
Meat to fat ratio	mean	4.41	4.08	4.28	4.58	4.54*	4.14
	CV	12.35	14.63	16.99	10.55	9.76	16.65
Meat to bone ratio	mean	5.49	5.49	5.43	5.43	5.30	5.63**
	CV	10.96	5.33	5.41	7.70	6.38	4.63

Means followed by ** and * are significantly different at $\alpha = 0.01$ and $\alpha = 0.05$, respectively

Carcass dressing percentage was comparable in all groups, ranging from 76.21% (group I) to 77.39% (group IV, Table 5). Dietary treatments had no effect on the meat to fat ratio and the meat to bone ratio (Table 5). Similar results were obtained by DENLI et al. (2005) in an experiment on quails. BONOMI et al. (2002) noted a significant increase in carcass muscle content and dressing percentage in ducks fed a diet supplemented with 40 ppm propolis, compared with birds that received 20 ppm propolis. In a study by KLECZEK et al. (2007), broiler chickens fed a propolis-supplemented diet were characterized by a significantly higher carcass dressing percentage, in comparison with birds fed a non-supplemented diet.

The weight of lean meat, bones and giblets was highly significantly higher in males than in females (Table 5). The weight of skin with fat was similar in males and females, at approximately 300 g. Carcass lean content was only 0.75% higher in males than in females. Females, compared with males, had a higher carcass fat content (16.73% vs. 15.21%). Carcass bone content was

significantly higher in males than in females. The percentage content of giblets in the carcass oscillated around 4.00% in both males and females. The meat to fat ratio was more desirable in males than in females (4.54:1 vs. 4.14:1), whereas the meat to bone ratio was highly significantly higher in females than in males (5.63:1 vs. 5.30:1, Table 5).

There were no significant differences between groups in the weight and percentage content of lean meat, muscles, skin with fat and bones in the breast (Table 6). However, breast composition was more desirable (higher muscle content, lower fat content) in chickens fed 50 mg propolis per kg feed, compared with the other groups. Also in a study by KLECZEK et al. (2007), broilers fed diets supplemented with propolis had the highest proportion of breast muscles in the carcass.

Table 6

Weight [g] and content [%] of tissue components in the breast of chickens aged 6 weeks

Specification	Statistics	Group				Gender	
		I	II	III	IV	♂	♀
Weight [g] of:							
lean meat	mean	584.7	602.3	616.0	610.3	649.5**	557.2
	CV	14.42	9.04	16.13	13.94	10.92	10.73
including:	mean	524.3	533.6	535.9	542.8	571.8**	496.5
breast muscles	CV	15.44	8.66	15.25	15.25	11.26	11.35
skin with fat	mean	78.7	88.2	89.8	80.3	86.8	81.6
	CV	22.77	18.28	28.88	21.65	17.07	30.54
bones	mean	40.3	37.2	38.3	37.25	42.9**	33.6
	CV	13.28	13.50	25.96	22.20	15.72	13.22
Content [%] in the breast:							
lean meat	mean	82.03	81.84	82.11	83.05	82.43	82.09
	CV	2.21	2.54	2.57	1.69	1.57	2.83
breast muscles	mean	73.49	72.53	71.59	73.74	72.58	73.09
	CV	4.33	3.08	4.47	2.33	4.17	3.17
skin with fat	mean	10.96	11.97	11.86	10.91	11.01	11.84
	CV	15.36	15.78	23.74	17.03	12.26	23.27
bones	mean	5.70	5.05	5.12	5.07	5.47*	4.99
	CV	11.71	10.10	21.51	13.39	13.37	15.07

Means followed by ** and * are significantly different at $\alpha = 0.01$ and $\alpha = 0.05$, respectively

The weight [g] and percentage content of tissue components in the legs are presented in Table 7. The weight of lean meat, skin with fat and bones in the legs was not significantly affected by the diet, and the percentage content of the above components in total leg weight varied between groups. The legs of chickens fed 50 mg propolis had a significantly higher muscle content and a lower fat content, compared with the legs of birds fed a diet with AGP (72.65% vs. 68.68% and 14.68% and 18.12%, respectively). The weight of lean

meat and bones in the legs was significantly higher in males than in females (Table 7). The percentage content of skin with fat in the legs was considerably higher in females than in males (17.49% vs. 15.05%). The lean meat content of the legs was comparable in males and females (approximately 71%), and the bone content of the legs was significantly higher in males than in females (Table 7).

Table 7

Weight [g] and content [%] of tissue components in the legs of chickens aged 6 weeks

Specification	Statistics	Group				Gender	
		I	II	III	IV	♂	♀
Weight [g] of:							
lean meat	mean	459.3	418.4	448.8	466.2	504.4**	392.0
	CV	18.71	13.03	18.54	15.53	11.70	9.99
skin with fat	mean	99.9	109.3	105.56	94.2	106.5	98.2
	CV	18.36	15.01	23.70	20.22	17.75	23.00
bones	mean	76.4	74.4	76.9	75.6	88.4**	63.3
	CV	19.10	16.68	24.61	22.20	12.45	9.69
Content [%] in the legs:							
lean meat	mean	71.40^{ABac}	68.68^{Bb}	70.58^{ABbc}	72.65^{Aa}	71.57	70.09
	CV	2.95	3.79	4.43	3.03	3.22	4.74
skin with fat	mean	15.66^{ABbc}	18.12^{Aa}	16.62^{ABac}	14.68^{Bb}	15.05	17.49**
	CV	14.74	17.12	19.28	13.62	11.88	19.77
bones	mean	11.93	12.20	12.02	11.71	12.58**	11.34
	CV	9.66	10.43	10.42	10.90	9.33	7.59

Means followed by capital letters or ** are significantly different at $\alpha = 0.01$, means followed by small letters or * are significantly different at $\alpha = 0.05$

Conclusions

The results of this study indicate that the growth performance of broilers fed a diet supplemented with 50 mg propolis was comparable to the growth performance of chickens fed a diet with AGP and slightly better than that of birds that received a non-supplemented diet. Contrary to expectations, propolis had no significant beneficial influence on the body weights and carcass dressing percentage of chickens, which could result from too low doses of the supplement. Since there is a scarcity of published research on dietary propolis supplementation in poultry, the present study may pave the way for further investigations involving higher dietary inclusion levels of propolis, at 100–500 mg kg^{-1} feed.

Translated by Aleksandra Poprawska

Accepted for print 1.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 119–127, Y. 2014

THE EFFECT OF DIETARY SUPPLEMENTATION WITH A HERBAL PRODUCT, A BLEND OF ORGANIC ACIDS AND ZINC OXIDE ON NUTRIENT DIGESTIBILITY AND GROWTH PERFORMANCE IN WEANED PIGLETS

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K e y w o r d s: herbal product, organic acids, zinc oxide, nutrient digestibility, growth performance, weaned piglets.

A b s t r a c t

The objective of this study was to determine the effect of dietary supplementation with a blend of organic acids, zinc oxide and the B-Safe® herbal product on nutrient digestibility, nitrogen balance and growth performance in weaned piglets. Nutrient digestibility was determined by a simple balance method, on 24 weaners (PIC) with average body weight of 28 kg, divided into four groups of six animals each. A five-day experimental period was preceded by a seven-day adjustment period. A production trial was carried out on 1279 weaned piglets that were fed four experimental diets: a control diet without feed additives (*A*), a diet supplemented with a blend of organic acids at 5 kg t^{-1} (*B*), a diet supplemented with zinc oxide at 3 kg t^{-1} (*C*), and a diet supplemented with the B-Safe® herbal product at 3 kg t^{-1} (*D*). The experiment lasted 19 days. A mashed starter diet was offered *ad libitum*. The body weights of piglets and feed intake were determined at the beginning and at the end (day 19) of the experiment.

The inclusion of an organic acid blend, zinc oxide and the B-Safe® herbal product in weaner diets highly significantly improved the digestibility of crude protein, crude fat ($P \leq 0.01$) and organic matter ($P \leq 0.05$). Nitrogen retention was higher in weaners fed a diet supplemented with zinc oxide at 3 kg t^{-1} (group *C*) than in control group animals (20.15 vs. 17.59 g, $P \leq 0.01$). The feed conversion ratio (FCR) was highly significantly lower in weaned piglets fed zinc oxide or B- Safe® at 3 kg t^{-1}, compared with the control group (1.50 and 1.47 vs. 1.70 kg kg^{-1}).

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WPŁYW DODATKU PREPARATU ZIOŁOWEGO, MIESZANINY KWASÓW ORGANICZNYCH I TLENKU CYNKU NA STRAWNOŚĆ SKŁADNIKÓW POKARMOWYCH ORAZ WYNIKI ODCHOWU WARCHLAKÓW

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S ł o w a k l u c z o w e: dodatki ziołowe, kwasy organiczne, tlenek cynku, strawność składników pokarmowych, wyniki odchowu, warchlaki.

A b s t r a k t

Celem doświadczenia było określenie wpływu dodatków: mieszaniny kwasów organicznych, tlenku cynku i preparatu B-Safe® na strawność składników pokarmowych, bilans azotu oraz wyniki produkcyjne warchlaków. Strawność składników pokarmowych określono za pomocą bezpośredniej metody bilansowej, na 24 warchlakach (PIC) o średniej masie ciała 28 kg, podzielonych na 4 grupy, po 6 sztuk w każdej. Pięciodniowy właściwy okres doświadczalny poprzedzono siedmiodniowym okresem przygotowawczym. Badaniami produkcyjnymi objęto 1279 warchlaków, u których zastosowano cztery mieszanki paszowe eksperymentalne: kontrolną bez dodatków paszowych (*A*), tę samą mieszankę paszową z dodatkiem kwasów organicznych, w ilości 5 kg t^{-1} (*B*), tlenek cynku w ilości 3 kg t^{-1} (*C*) i preparat B-Safe® w ilości 3 kg t^{-1} (*D*). Badania trwały 19 dni. Mieszankę starter zastosowaną w doświadczeniu wyprodukowano w formie sypkiej i podawano prosiętom *ad libitum*. Na początku i na końcu doświadczenia (19 dzień) mierzono masę ciała warchlaków. Analizowano również ilość pobranej mieszanki paszowej.

Suplementacja dawki pokarmowej dla warchlaków dodatkiem: mieszaniny kwasów organicznych, tlenku cynku oraz preparatu B-Safe® wysoce istotnie poprawiła strawność białka ogólnego, tłuszczu surowego ($P \leq 0{,}01$) i substancji organicznej ($P \leq 0{,}05$). Stwierdzono wysoce istotną (20,15 vs 17,59 g, $P \leq 0{,}01$) poprawę retencji azotu u zwierząt otrzymujących w diecie dodatek tlenku cynku (*C*) w ilości 3 kg t^{-1} w stosunku do warchlaków z grupy kontrolnej. Świnie otrzymujące dodatek tlenku cynku lub produktu B-Safe® w ilości 3 kg t^{-1} charakteryzowały się wysoce istotną poprawą wykorzystania paszy na kg przyrostu (1,50; 1,47 vs 1,70 kg kg^{-1}) w stosunku do zwierząt z grupy kontrolnej.

Introduction

A ban on the use of antibiotic growth promoters (AGPs) in animal feed entered into effect in the European Union on 1 January 2006 (VONDRUSKOVA et al. 2010). The ban was introduced, among others, due to concerns about the emergence of multiple drug-resistant bacteria in the digestive tract of farm animals and the possibility of cross-resistance with therapeutic antibiotics used in humans (RIEMENSPERGER et al. 2012, THACKER 2013). The economic effects of the ban on AGPs in livestock production include reduced growth rate and deterioration in animal health (CASEWELL et al. 2003, GRELA and SEMENIUK 2006). Recent years have witnessed an increasing interest among food producers, animal breeders and farmers in natural feed additives as alternatives to AGPs (VERSTEGEN and WILLIAMS 2002). Growing consumer

awareness of food safety and quality issues, including the health benefits and quality of animal products, is also an important consideration (MONTESISSA and CALINI 2006).

Feed additives used in pig diets include organic acids, phytobiotics and microelements such as zinc (THACKER 2013), which can positively affect gut microbiota thus improving nutrient digestibility and increasing productivity (PETTIGREW 2006, VONDRUSKOVA et al. 2010). Organic acids are found naturally in herbs and animal tissues. They exert bacteriostatic effects by maintaining a low pH of the digesta, which prevents the colonization of the gastrointestinal tract by pathogens (KIL et al. 2006, COSTA et al. 2013). They also reduce the risk of diarrhea and improve nutrient utilization (LALLES 2008, GERRITSEN et al. 2010). Zinc oxide has also been shown to increase daily gains and reduce the incidence of intestinal diseases in weaned piglets (SHELTON et al. 2011, PIEPER et al. 2012, HU et al. 2013). Herbal extracts improve nutrient digestibility and utilization, and exhibit antibacterial and antioxidant properties thus increasing livestock production efficiency (WANG et al. 2007, LIU et al. 2008, COSTA et al. 2013).

The aim of this study was to determine the effect of dietary supplementation with a blend of organic acids, zinc oxide and the B-Safe® herbal product on growth performance and nutrient digestibility in weaned piglets.

Materials and Methods

The experiment was conducted on a commercial pig farm. The experimental materials comprised weaned piglets of a PIC line, divided into four groups with eight replicates per treatment and 34 to 44 animals per replicate. The weaners were kept on a slatted floor, in a building equipped with an automatic feeding system and mechanical ventilation. The animals had free access to feed and water. After weaning, the piglets were weighed and allocated to dietary treatment groups by body weight and sex.

The weaners were fed a mashed starter diet formulated to meet their nutrient requirements (Table 1). The control diet contained maize grain, wheat grain, barley grain, soybean meal, soybean oil, Specilac (a product containing modified soybean protein), and a premix. The diet fed to control group *A* did not contain feed additives affecting gut microflora. The diet for experimental group *B* was supplemented with a blend of organic acids (propionic, formic, fumaric, lactic and citric) and their salts at 5 kg t^{-1} feed. The diet for experimental group *C* was supplemented with zinc oxide at 3 kg t^{-1} feed. Group *D* animals were fed a diet supplemented with the B-Safe® herbal product (3 kg t^{-1} feed) that contains plant extracts and synthetic clays

(*Satureja montana* leaves, chestnut tannins, *Trigonella foenum graecum*, mineral clays (sepiolite & zeolite), copper sulfate, vegetable oil, limestone, dextrose, soft wheat white shorts, clays (E 562, E554) and chicory pulp as processing aids).

Table 1

Composition and nutritional value of the control diet

Specification	Starter
Maize [%]	25.0
Wheat [%]	20.0
Barley [%]	15.0
Soybean meal [%]	24.0
Specilac (soybean replacer) [%]	5.0
Soybean oil [%]	1.0
Starter premix* [%]	10.0
Nutritional value	
Metabolizable energy [MJ/kg]	13.6
Crude protein [%]	20.5
Lysine [%]	1.36
Methionine+cystine [%]	0.76
Threonine [%]	0.84
Tryptophan [%]	0.25
Calcium [%]	0.80
Digestible phosphorus [%]	0.40
Sodium [%]	0.15

* crude protein – 12.5%, Ca – 5.8%, P – 2.1%, Na – 1.5%, lysine – 3.6% methionine – 1.2%, threonine – 1.4%, lactose – 25.0%, Vit. A – 200 000 IU, Vit. D_3 – 20 000 IU, Vit. E – 1 400 mg, Vit. K_3 – 30 mg, Vit. B_1 – 30 mg, Vit. B_2 – 80 mg, Vit. B_6 – 60 mg, Vit. B_{12} – 0.5 mg, niacin 400 mg, pantothenic acid – 200 mg, folacin – 40 mg, biotin – 2 mg, choline chloride – 6 000 mg, Zn – 1 400 mg, Mn – 800 mg, Cu – 1 600 mg, Fe – 1 500 mg, J – 12 mg, Co – 6 mg, Se – 3 mg, antioxidant (+), xylanase + β-glucanase + phytase (+)

The body weights of piglets and feed intake were determined at the beginning and at the end (day 19) of the experiment. The data were used to calculate feed conversion ratio (FCR) measured as kg feed intake per body weight gain. The nutrient content of diets was determined by the Weende analysis (AOAC 2000). The chemical composition of diets is presented in Table 2.

Table 2

Chemical composition of experimental diets

Specification	Diets			
	A	*B*	*C*	*D*
Dry matter [%]	88.96	88.65	88.69	88.87
Crude ash [%]	4.93	5.37	5.45	4.74
Crude protein [%]	21.25	21.71	21.08	21.27
Ether extract [%]	4.32	4.31	4.25	3.94
Crude fiber [%]	3.02	2.86	3.02	2.65
N-free extracts [%]	55.44	54.4	54.89	56.27

Nutrient digestibility was determined by a simple balance method, on 24 weaners with average body weight of 28 kg, divided into four groups of six animals each. The animals were kept in individual metabolism cages. A five-day experimental period was preceded by a seven-day adjustment period. The weaners were fed twice daily, at 7.00 a.m. and 3.00 p.m., and they had free access to drinking water.

The results were processed statistically by one-way ANOVA and Duncan's test. Arithmetic means (x), standard errors of the mean (SEM) and significance level (P) were determined. All calculations were performed using STATISTICA 10 software.

Results and Discussion

The coefficients of nutrient digestibility are shown in Table 3. Dietary supplementation with a blend of organic acids, zinc oxide and the B-Safe® herbal product had a statistically significant effect on nutrient digestibility. The tested supplements highly significantly ($P \leq 0.01$) improved the digestibility of total protein (by 2.5, 2.6 and 3%, respectively) and crude fat (by 19.6, 23.8, 24.7%, respectively), compared with the control group. No significant differences were noted between groups in the digestibility of crude fiber and N-free extractives. Different results were reported by GERRITSEN et al. (2010) who supplemented weaner diets with a blend of organic acids (formic, propionic, lactic, citric and sorbic). The cited authors demonstrated that the diet containing organic acids improved crude fiber digestibility, in comparison with the control diet (25.7 vs. 22.7%, $P \leq 0.01$), but organic acids had no effect on the digestibility of crude protein and crude fat. In a study by HAN and THACKER (2009), the digestibility of crude protein and dry matter did not increase in response to zinc oxide added to weaner diets at 1500 mg kg^{-1} and 2500 mg kg^{-1}.

In our study, the digestibility of organic matter improved significantly in weaned piglets fed diets with the tested feed additives (83.0; 83.0; 83.1 vs. 82.0%, $P \leq 0.05$). An increase in organic matter digestibility in pigs weighing 22 to 45 kg fed diets supplemented with lactic acid and formic acid (82.47; 82.66 vs. 81.80% $P \leq 0.05$) was also reported by JONGBLOED et al. (2000).

An analysis of nitrogen balance (Table 3) revealed that zinc oxide at 3 kg t^{-1} contributed to a highly significant increase in nitrogen retention, in comparison with the control group (20.15 vs. 17.59 g). Diet supplementation with zinc oxide increased the efficiency of nitrogen utilization relative to nitrogen intake and digestion, but the noted differences were statistically non-significant.

Table 3

Apparent fecal digestibility coefficients and N-balance

Specification	Control *A*	Organic acids *B*	Zinc oxide *C*	B-Safe® *D*	SEM	*P*
Digestibility coefficients	–	–	–	–	–	–
Crude protein [%]	75.9^{B}	78.4^{A}	78.5^{A}	78.9^{A}	0.22	0.006
Ether extract [%]	55.1^{B}	74.7^{A}	78.9^{A}	79.8^{A}	0.55	0.004
Crude fiber [%]	36.8	41.2	38.8	40.8	0.25	0.234
N-free extractives [%]	89.2	88.5	89.3	88.5	0.29	0.291
Organic matter [%]	82.0^{b}	83.0^{a}	83.0^{a}	83.1^{a}	0.22	0.002
Nitrogen balance	–	–	–	–	–	–
Retention [g]	17.59^{B}	18.83	20.15^{A}	18.65	0.12	0.009
N utilization/N intake [%]	45.9	46.2	49.7	46.4	0.35	0.125
N utilization/N digestion [%]	61.0	58.0	63.7	61.6	0.48	0.339

a, b – $P \leq 0.05$

A, B – $P \leq 0.01$

The results of a growth trial are summarized in Table 4. The average initial body weight of weaned piglets in all groups was around 9.5 kg. After 20 days of the experiment, higher body weights were determined in animals fed diets supplemented with a blend of organic acids (16.54 kg), zinc oxide (17.17 kg) and the B-Safe® herbal product (17.08 kg), compared with the control group, but the observed differences were not confirmed by a statistical analysis. Body weight gains tended to increase in experimental groups, in comparison with the control group (380, 404 and 395 vs. 355 g/day). Despite an absence of significant differences between groups, the inclusion of zinc oxide and B-Safe® in weaner diets contributed to higher weight gains.

Table 4

Growth performance of weaned piglets during the starter phase

Specification	Control 5 kg t^{-1}	Organic acids 3 kg t^{-1}	Zinc oxide 3 kg t^{-1}	B-Safe® 3 kg t^{-1}	SEM	*P*
No. of pigs	319	312	328	320		
Initial body weight [kg]	9.50	9.38	9.37	9.59	0.181	0.975
Final body weight [kg]	16.51	16.54	17.17	17.08	0.358	0.884
Feed intake [g d^{-1}]	580	570	589	565	11.939	0.906
Daily gain [g d^{-1}]	355	380	404	395	9.448	0.283
FCR [kg kg^{-1}]	1.70^{Aa}	1.53^{b}	1.50^{B}	1.47^{B}	0.026	0.004
Mortality [%]	2.53	1.56	1.89	1.57	0.408	0.834

a,b – $P < 0.05$

Our results corroborate the findings of HU et al. (2013) who observed a significant difference in final body weight between weaners fed zinc oxide at 2250 mg kg^{-1} for 14 days and control group animals (10.11 vs. 9.62 kg, $P \leq 0.05$); the average initial body weight of piglets was 6.12 kg. In a study by RIEMENSPERGER et al. (2012), piglets weaned at 28 days of age were fed a diet supplemented with a blend of formic, acetic and propionic acids, which resulted in a highly significant difference in final body weight (day 56) between experimental and control animals (37.71 kg vs. 35.54 kg, $P \leq 0.01$). There was also a significant difference in daily gains, determined during the entire experiment, between weaners fed a blend of organic acids and their control counterparts (516 vs. 483 g/day). SHELTON et al. (2011) observed a significant difference in final body weight (day 42 of the experiment) between weaned piglets receiving zinc oxide at 3,000 mg kg^{-1} and control group animals (26.2 vs. 24.6 kg, $P \leq 0.05$). The above authors noted also a significant ($P \leq 0.05$) difference in average daily gains, which reached 473 and 440 g in the experimental and control group, respectively.

In the present study, average daily feed intake was comparable in all animals, at 580 g in the control group, 570 g in group *B* fed a diet supplemented with organic acids, 589 g in group *C* fed zinc oxide, and 565 g in group *D* that received the B-Safe® herbal product.

Control group animals were characterized by the highest FCR – 1.70 kg kg^{-1}. The inclusion of organic acids in weaner diets (group *B*) improved FCR (1.53 vs. 1.70 kg kg^{-1}, $P \leq 0.05$), compared with the control group. The lowest FCR was noted in piglets fed zinc oxide (group *C*) and B-Safe® (group *D*) at 3 kg t^{-1} (1.53 and 1.48 vs. 1.70 kg kg^{-1}, $P \leq 0.01$). HAN and THACKER (2010) demonstrated that FCR decreased in weaned piglets fed 1500 mg kg^{-1} ZnO, but the difference relative to the control group was not statistically significant (1.50 vs. 1.55 kg kg^{-1}). RIEMENSPERGER et al. (2012) did not observe significant differences in FCR between groups, either, but in their study FCR was lower (1.99 kg kg^{-1}) in weanlings fed an organic acid blend than in animals fed a diet without feed additives (2.04 kg kg^{-1}).

Dietary treatments had no effect on the health status and mortality rates of weaned piglets.

Conclusions

The inclusion of an organic acid blend, zinc oxide and the B-Safe® herbal product in weaner diets highly significantly improved the digestibility of crude protein, crude fat ($P \leq 0.01$) and organic matter ($P \leq 0.05$). Nitrogen retention was highly significantly higher in weaners fed a diet supplemented with zinc

oxide at 3 kg t^{-1} (group *C*) than in control group animals (20.15 vs. 17.59 g, $P \leq 0.01$). FCR was highly significantly lower in weaned piglets fed zinc oxide or B-Safe® at 3 kg t^{-1}, compared with the control group (1.50 and 1.47 vs. 1.70 kg kg^{-1}).

Translated by Aleksandra Poprawska

Accepted for print 1.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 129–135, Y. 2014

ERRORS IN IDENTIFYING COAT COLOURS IN HORSES: THE SCALE OF THE PROBLEM

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K e y w o r d s: coat colour, horses, identification, colour inheritance pattern.

A b s t r a c t

The identification system of horses used in Poland is based on the old classification of coat colours which does not include all colours based on colour inheritance patterns. As a result, some colours are incorrectly described. In order to evaluate the scale of the problem, the accuracy of colour identification was assessed by verifying 5779 pedigrees of horses of three different breeds. The verification was performed based on the colour inheritance patterns using a classification of discrepancies. Such discrepancies were detected in 26 cases and related mainly to diluted colours and black. The problem, however, should not be neglected. The number of incorrectly described horses is probably higher, since not all discrepancies can be detected based only on breeding records. The study demonstrated that some errors could be avoided by updating the classification of coat colours. However, a complete elimination of errors is impossible without genetic testing.

BŁĘDY W IDENTYFIKACJI UMASZCZENIA U KONI – SKALA PROBLEMU

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S ł o w a k l u c z o w e: umaszczenie, konie, identyfikacja, dziedziczenie umaszczenia.

A b s t r a k t

Opis identyfikacyjny koni stosowany w Polsce oparty jest na przestarzałej klasyfikacji maści. Nie uwzględniane są wszystkie maści, których istnienie potwierdzono dzięki pogłębieniu wiedzy na temat zasad dziedziczenia umaszczenia koni. W efekcie część maści jest nieprawidłowo opisywana. W związku z tym, aby ocenić skalę problemu, przeprowadzono analizę poprawności identyfikacji

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maści, weryfikując 5779 rodowodów koni trzech różnych ras. Weryfikację prowadzono w oparciu o reguły dziedziczenia umaszczenia, wykorzystując opracowaną do tego celu klasyfikację nieprawidłowości. Takie nieprawidłowości stwierdzono w 26 przypadkach. Dotyczyły przede wszystkim maści rozjaśnionych oraz maści karej. Nie należy jednak lekceważyć problemu. Liczba błędnie opisanych koni jest najprawdopodobniej większa, ponieważ nie wszystkie nieprawidłowości są możliwe do wykrycia w oparciu wyłącznie o dokumentację hodowlaną. W badaniach wykazano, że można uniknąć części błędów poprzez uaktualnienie klasyfikacji maści. Jednakże całkowite wyeliminowanie błędów nie jest możliwe bez zastosowania testów genetycznych.

Introduction

Before humans started to interfere in the selection of horses for breeding, primitive horses had a relatively limited variety of colours. The majority of them were blue dun, black, dun, bay or brown, which had been a specific camouflage for this species in its natural environment (CZAPSKI 1874, STACHURSKA et al. 2001). Differently-coloured specimens were usually been eliminated by means of natural selection (CHACHUŁA et al. 1991, GRANDIN and DEESING 1998). When the horse was domesticated, humans started to implement a selection process which included varied traits such as coat colours. Horses with rare colours, which initially would have had fat chance for survival, such as skewbald, grey or palomino, were favoured by humans. The selection of these horses for breeding provoked an elevated frequency of initially rare alleles, which resulted in a higher variety of colours in this species (CHACHUŁA et al. 1991). Today, there are over 30 known colours (STACHURSKA 2002). Coat colours in horses as a typical trait are used to identify these animals and, therefore, accurate description of the colours is very important. Moreover, some breeders wish to have a specific colour of offspring, which is only possible with a precise and accurate description of parental colours. Knowing the colour of parents and offspring, it is possible to eliminate or question the origin of a horse by using the colour inheritance pattern (MIKULSKA 1999).

In Poland, the identification of horse colours is based on the phenotype, usually without taking in consideration the patterns of its inheritance. In addition, an identifying description uses the traditional classification of colour, which does not include such colours as buckskin or red-dun (STACHURSKA 2002). This situation facilitates errors in determining the colours of horses. Therefore, in order to determine a scale of the problem and types of errors, it was attempted to verify the accuracy of colour identification in the selected horse breeds by using the inheritance patterns.

Material and Methods

To analyse the accuracy of colour identification, 5770 horse pedigrees were verified. The data originated from the following stud books:

– the 1st and 2nd volume of The Stud Book of Polish Halfbred Horses (Ksp) – 2160 horses,

– the 1st and 2nd part of volume 4 of The Stud Book of Wielkopolska Horses (Kwlkp) – 2382 horses,

– the 4th volume of The Stud Book of Malopolska Horses (Km) – 1237 horses.

The errors in colour descriptions were identified based on the assumed inheritance patterns and according to up-to-date genetic knowledge by comparing the colour of offspring with the colours of the parents. The verification procedures included three generations of horses. The discrepancies were classified and presented in tables. A personal classification was formulated to systematize the data:

1) Discrepancies based on the properties of alleles located on the locus A (Agouti): the offspring of bay or brown (A_E_) cannot originate from mating black coloured horses (aaE_) and blue dun individuals (aaE_D_) (the lack of allele A in the genotype);

2) Discrepancies in diluted colours determined by the genes located on the locus C (cream colours) and on the locus D (dun colours):

– the offspring of palomino horses (_ _eeCC^{cr}dd) cannot originate from the mating of horses in which none of individuals is cream diluted (the lack of allele C^{cr} in the genotype),

– the offspring of dun horses (A_E_D_) cannot originate from the mating of horses in which none of the individuals is dun diluted (the lack of allele D in the genotype).

3) Discrepancies based on the dominant nature of allele G which determines grey colour: the offspring grey in colour (_ _G_) cannot originate from the mating of horses in which none of the individuals was grey (the lack of allele G in the genotype).

Results and Discussion

The analyses of the accuracy of horse colours based on the patterns of inheritance revealed discrepancies in 26 cases, which constituted 0.45% of all pedigrees included in the study (Table 1). The majority were discrepancies of type 2, i.e. related to the identification of diluted colours (13 cases) and of type 1, i.e. related to the crossbreeding of black horses (10 cases). The most mistakes

were detected in the descriptions of Wielkopolska horses (14 discrepancies) and only in this breed were type 3 discrepancies (related to the identification of grey colour) found (3 cases).

Table 1
Discrepancies detected in pedigrees of horses of selected breeds with regard to the coat colour

Breed	*n*	Discrepancies						Total	
		1		2		3			
		n	[%]	*n*	[%]	*n*	[%]	*n*	[%]
Sp*	2160	5	0.23	2	0.09	0	0.00	7	0.32
Wlkp**	2382	3	0.13	8	0.34	3	0.13	14	0.59
Młp***	1237	2	0.16	3	0.24	0	0.00	5	0.40
Total	5779	10	0.17	13	0.22	3	0.05	26	0.45

* Polish Halfbred Horse
** Wielkopolska Horse
*** Malopolska Horse

The discrepancies of type 2 were detected based on the dominant nature of alleles C^{Cr}, and alleles D which determine the occurrence of diluted cream and dun colours by the specific dilution of basic colours. The presence of allele C^{cr} in the genotype of at least one parent is a prerequisite for obtaining an offspring with cream dilution. Similarly, the presence of allele D is a prerequisite for obtaining an offspring with dun dilution. In other words, due to the dominant nature of these alleles, at least one of the parents must be diluted in colour (STACHURSKA 2002). However, it was found in the verified pedigrees that the parents without these alleles in the genotype produced diluted offspring (palomino or dun). In this group, the most common discrepancies were associated with a situation in which the mating of a dun or golden bay with a chestnut or bay horse produced palomino offspring or vice versa: dun offspring were produced by mating a palomino horse with a bay individual. Since the palomino and dun colours are determined by separate genes, a dun sire (A_E_ CCD_) cannot pass the allele C^{cr} which determines palomino colour to its offspring, and a palomino parent (_ _eeddCCCr) cannot pass the allele D which determines dun colour. Similarly, these alleles cannot be transferred by a parent of basic colours (STACHURSKA and ZASADNY 1999). These discrepancies can have different causes. It seems most probable that the horses identified as dun were actually of buckskin colour (A_E_CC^{Cr}dd), which would explain the presence of C^{cr} allele in the offspring. Since the buckskin colour is not featured in the colour classification used in the identification of the analysed horse breeds, the horses of this colour are mistakenly described as dun, golden bay or light bay. These colours are phenotypically very similar to buckskin colour,

which is characterized by a yellowish-brown coat colour, black mane and tail as well as black markings on the legs (STACHURSKA 2002).

The abnormalities of type 2 could have also resulted from an erroneous identification of a "chestnut" parent which was actually palomino (_ _eeCC^{Cr}dd) or vice-versa, aǹ palomino' offspring was light chestnut (sorrel) (_ _eeCCdd). It cannot be excluded that a dun parent was genotypically buckskin-dun (A_E_CC^{Cr}D_), although this case seems less likely as the frequency of these colours is very low in the populations of the analysed breeds.

Two palomino offspring were detected as having originated from two chestnut parents. Since chestnut horses (_ _eeCCdd) cannot pass the C^{cr} allele to their offspring, they cannot produce palomino offspring. Moreover, horses of this colour crossed with each other can only produce chestnut offspring, which results from the epistatic nature of the recessive ee alleles. The reason for this abnormity was most probably the mistaken description of a palomino horse as light-chestnut. These colours are very similar. In both colours, horse hair is very fair, almost white. The coat of a palomino horse may range from fair-cream to deep golden, whereas light chestnut horses have fair yellow-red coat (CASTLE and SINGLETON 1961, STACHURSKA 2002).

Type 1 discrepancies were relatively common and related to the mating of two black horses (aaE_) and two blue dun horses (aaE_D_). Such mating cannot produce any bay foal (A_E_) as parents cannot transfer the allele A. The most probable explanation of this abnormity seems to be an erroneous description of one of the parents or the offspring itself. Black, dark-bay and sometimes brown colours are often very similar, which hinders a correct colour identification (STACHURSKA et al. 2004). Therefore, one of black parents could have been genotypically bay or brown, or dark-bay offspring could have been actually black. The occurrence of bay individuals can be also explained by the hypothesis that a dominant black colour (E^{D}) may exist in which the presence of allele A would be unnoticeable (DREUX 1966, SPONENBERG and WEISE 1997). The majority of studies carried out to date have rejected the existence of such an allele and colour (MIKULSKA 1999, RIEDER et al. 2001, STACHURSKA 2002).

Although the background of the aforementioned discrepancies may be explained relatively easily, it does not apply to type 3 mating, in which none of the parents of a grey individual was grey in colour. This situation was detected three times while analysing the pedigrees of the Wielkopolska horse breed. The presence of allele G causes greying in the horse, regardless of the initial colour (except for white dominant colour). If an individual has this allele, it is thus visible in the phenotype. Such an error could be relatively easily explained if made in a description of foals or younger horses (as the symptoms of greying may not be visible), but it is much more difficult to be justified in horses registered in stud books. Horses are entered in a stud book at the age of app.

three years when the signs of greying are clearly visible. Therefore, any discrepancies in the description are probably caused by human error (e.g. while copying information into a stud book). As the inheritance pattern of grey colour has been known for long (ZWOLIŃSKI 1977), it seems even more surprising that a person who recorded the data did not question the origin of such horse or the accuracy of its parents' description. It indicates that persons authorized to record the description of horses have limited knowledge on the genetic background of colour inheritance pattern.

Even though the above-mentioned errors may seem trivial, for a person who understands the colour inheritance patterns, a discrepancy in a description may constitute a basis for questioning the origin of such horses. Despite the percentage of discrepancies in the description being relatively low (0.45%), they should not be neglected. The number of erroneously described horses is presumably much higher, taking into consideration the fact that not all discrepancies can be detected based only on breeding records. For instance, the frequency of buckskin horses may be much higher, because these horses were often described as bay, and a colour of bay horse with a bay parent cannot be questioned.

Conclusions

Some of the detected discrepancies could be caused by mistaken parentage, but the genetic control of parentage which is now commonly conducted, will reduce the number of such mistakes. Most of the discrepancies indicate the errors in describing the coat colours in horses. The substantial majority of them resulted from a lack of possibility to correctly identify the colour as the identifying description is based on traditional classification of coat colours. Most errors would be avoided if the classification of colours used in the identification records was updated according to the current state of knowledge on the patterns of colour inheritance. The buckskin, reddun and other colours should be included in the classification. Persons responsible for identification records should know the patterns of colour inheritance, which would also limit the errors. However, a complete elimination of errors is impossible without genetic tests which verify the colours of horses. For instance, identifying black and dark-bay or buckskin colours is sometimes difficult. Unfortunately, the wide-scale implementation of such tests seems unreal, at least for financial reasons.

Translated by JOANNA JENSEN

Accepted for print 3.09.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 137–152, Y. 2014

THE EFFECT OF ENVIRONMENTAL FACTORS ON THE STRUCTURE OF PHYTOPLANKTON IN THE LOWER ODRA RIVER

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K e y w o r d s: phytoplankton, large river, cooling water, power plant.

A b s t r a c t

Comparison of phytoplankton composition from selected sites in the lower Odra River was done to determine whether the effects of heated water are strongest, from the considered environmental factors. Samples of phytoplankton were collected in April, July and October 2009–2011 at five sites along the lower section of the Odra River. The most pronounced differences between the phytoplankton at the sites were revealed in the phytoplankton abundance and they were related to the time of water retention, the washing out of plankters from slack waters, and the predation by molluscs and zooplankton. The strongest correlations were found between the phytoplankton abundance, the content of inorganic nutrients and temperature. Taxonomic composition of phytoplankton at all sites in the same months was similar. Cooling water from the power plant seems to accelerate eutrophication in the discharge but has no significant impact on the phytoplankton composition downstream in the Odra River.

WPŁYW CZYNNIKÓW ŚRODOWISKOWYCH NA STRUKTURY FITOPLANKTONU W DOLNEJ ODRZE

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Abstrakt

Porównano skład fitoplanktonu z wybranych stanowisk dolnej Odry w celu określenia, który spośród badanych czynników środowiskowych ma najsilniejszy wpływ na kształtowanie owych struktur. Próby fitoplanktonu pobrano w kwietniu, lipcu i październiku w latach 2009–2011 na pięciu stanowiskach w dolnym odcinku Odry. Kompozycja taksonomiczna fitoplanktonu na wszystkich stanowiskach była podobna. Największe różnice w strukturach fitoplanktonu na poszczególnych stanowiskach dotyczyły jego liczebności. Było to związane z czasem retencji wody, wymywaniem fitoplanktonu z zastoisk oraz wyjadaniem tych organizmów przez mięczaki i zooplankton. Najsilniejsze korelacje zaobserwowano między liczebnością fitoplanktonu a zawartością nieorganicznych składników odżywczych i temperatury. Wody podgrzane z elektrowni wydają się przyspieszać proces eutrofizacji w kanale zrzutowym. Nie zaobserwowano jednakże istotnego wpływu podwyższonej temperatury wody na skład fitoplanktonu na stanowiskach zlokalizowanych poniżej ujścia kanału niosącego wody podgrzane.

Introduction

In lower sections of large rivers, the retention time can be long enough for plankton colonisation and reproduction (ALLAN 1998). Besides the hydrological conditions such as, time of retention and discharge, chemical (inorganic nutrients), physical (temperature, conductivity) and biotic (grazing, competition) are also important conditions for the growth of plankton structures (BASU and PICK 1997, MOSS et al. 1989, REYNOLDS 1988). With regards to plankton supply, of great significance is the presence of ponds, oxbow lakes or dam reservoirs permanently or periodically connected with the rivers (DEMBOWSKA 2009). The phytoplankton washed out from these water reservoirs into the rivers is often essential for development of the river phytoplankton (ALLAN 1998). The changes in phytoplankton composition also depends on the season and environmental conditions in a given different sections of that river (TAVERNINI et al. 2011), which offers specific conditions for the development of phytoplankton.

Because of the above described significant diversity in the sites, the lower section of the Odra River has been found suitable for study. It is characterised by many interconnected sections, offering different morphological and biological conditions, despite being in relatively close distance. We expect that phytoplankton assemblages at various sites would be different enough to form different communities. Moreover, exploring the forces responsible for those differences would be valuable in understanding the ecological dynamics in rivers.

A diversity of environmental conditions of the lower Odra sections, could relate to the anthropogenic activity, including the discharge of cooling water from a power plant. The effect of cooling water discharge on phytoplankton has been studied by many authors (JORDAN et al. 1983, ŁABĘCKA et al. 2005, POORNIMA et al. 2006, RAYMOND and RAYMOND 1969, TYSZKA-MACKIEWICZ

1983, ZARGAR and GHOSH 2006). The factors identified by most of them, as being responsible for changes in the phytoplankton composition include: mechanical stress, the lethal effect of temperature, the influence of chemical agents (used for conservation of cooling systems in a power plant) and predation by filter feeders. Although the effect of the temperature on phytoplankton has often been studied, the results are rather ambiguous. Reduced levels of phytoplankton production have been observed at the cooling water temperatures of 25°C [Lewis Creek Reservoir, Texas, (WELCH and WARD 1978)], 30°C [York River, Virginia, (WARINNER and BREHMER 1966); Lake Erie and Ontario, (CRIPPEN et al. 1978); mid-Atlantic power plants, (SMITH et al. 1974)], 37.5°C [Nanticoke Estuary, Maryland, (FLEMER and SHERK 1977)]. In Poland, the influence of cooling water on phytoplankton has been best recognised for the Power Plant Pątnów-Adamów-Konin (SOCHA and HUTOROWICZ 2009). However, these authors' studies concerned mainly limnic reservoirs. In general, there is not much literature from the central Europe region that pertains to the power plant effluent discharge and its effects. It would be practical for the specific remedial action.

According to DODDS (2006), phosphorus and nitrogen are the most important nutrients regulating the autotrophic state in running waters and their presence is positively correlated to gross primary production in the streams. The insufficient information on the influence of inorganic nutrients in the lower sections of rivers, such as the Odra River, has prompted this attempt to identify the factors influencing the phytoplankton growth.

Comparison of phytoplankton composition from selected sites in the lower Odra River was done to determine whether the effects of heated water are strongest, from the considered environmental factors. Realisation of the study required the following steps:

a) determination of abundance, similarity in qualitative and taxonomic composition of phytoplankton in the interconnected sections of the river;

b) determination of the influence of physico-chemical factors on the communities of river phytoplankton;

c) determination of the effect of cooling water on the phytoplankton composition in the main bed of the Odra River.

Methods

The Odra River is one of the major Central European lowland rivers (854 kilometres) and the second longest river in Poland. The lower Odra River is still classified as eutrophic although the status of its water has been improving (*Raport o stanie środowiska...* 2004). The samples were collected at

five different sites along the lower section of Odra River (Figure 1). The sites were selected taking into account conditions such as depth, width and the coverage of macrophytes. Site 1: the Odra River is 200 m wide, 10 m depth, before division, its bed is regular with bays overgrown by rushes; site 2: the West Odra River is 100 m wide, 7 m depth, upstream to this site, there is a weir regulating the water levels that form a reservoir; site 3: the point of discharge of the cooling water, from the power plant channel is 35 m wide, 4 m depth; site 4: the East Odra River, downstream of site 3, the river is about 175 m wide, 12 m depth, with a regular bed, and near the banks there is a narrow band of rushes; site 5: the Odyniec Channel joining the West and East sections of the Odra River, the banks have wide bands grown with rushes, the bottom is grown with macrophytes, the width of the channel at this site is close to 150 m and depth to 5 m, in which the rate of water flow is the lowest.

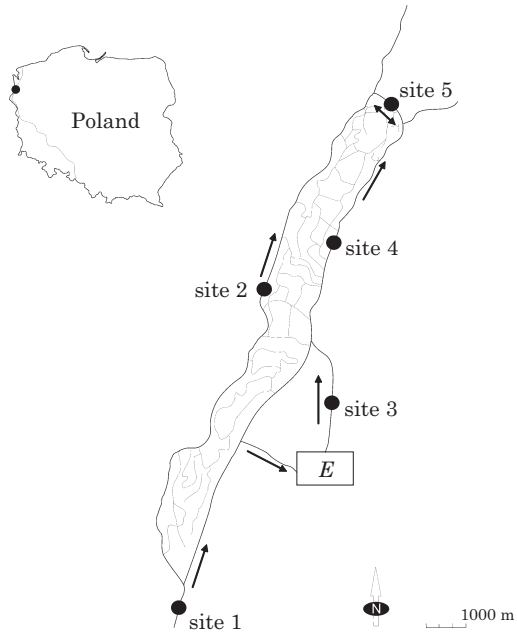


Fig. 1. The Odra River (study area) with sites. Site 1 – The Odra River before division, site 2 – The West Odra River, site 3 – The point of discharge of the cooling water, site 4 – The East Odra River, site 5 – The Odyniec Channel (joining the West and East sections of the Odra River), *E* – power plant, arrows – river flow

Samples of phytoplankton were collected in the months of April, July and October in the years 2009–2011. Each sample was taken just below the surface to fill the 1L containers. The samples were preserved in a 4% solution of formaldehyde. Phytoplankton subsample counting was made in 2 mL

Sedgewick-Rafter chambers. Identification was performed under a Nikon Eclipse 50i microscope. Identification of phytoplankton was made using taxonomic keys by STARMACH (1989), BUCKA and WILK-WOŹNIAK (2007), BURCHARD et al. (2010) to the lowest possible taxonomic unit. Enumerate algal forms were counted as one for each colony, filament, diatom cell (regardless if colonial or filamentous).

Temperature, pH, conductivity and dissolved oxygen content were measured by a CX-401 meter made by Elmetron. The contents of nitrites, nitrates, ammonium nitrogen, total nitrogen, total phosphorus and orthophosphates were measured by a Hach Lange DR-850 photometer. The mean values of the environmental variables of the seasons in 2009–2011 are given in Table 1.

Table 1
Mean values of physico-chemical variables at sites examined of the lower Odra in 2009–2011

	Temp. [°C]	pH	O_2 [mg dm^{-3}]	Cond. [μS cm^{-1}]	N-NH_3 [mg dm^{-3}]	N-NO_3 [mg dm^{-3}]	N-NO_2 [mg dm^{-3}]	P-PO_4 [mg dm^{-3}]	TN [mg dm^{-3}]	TP [mg dm^{-3}]
Site 1	16.0	8.35	8.03	629.1	0.12	0.7	0.011	0.20	1.9	0.46
Site 2	16.5	8.17	7.44	669.5	0.18	0.9	0.013	0.20	2.1	0.48
Site 3	22.3	8.27	7.33	744.6	0.15	0.9	0.012	0.21	2.0	0.50
Site 4	16.8	8.30	7.50	660.9	0.14	0.8	0.010	0.18	2.1	0.42
Site 5	16.7	8,46	8.68	651.4	0.18	0.8	0.019	0.18	2.0	0.46

Temp. – temperature, cond. – conductivity, N-NH_3 – ammoniacal nitrogen, N-NO_3 – nitrate nitrogen, N-NO_2 – nitrite nitrogen, P-PO_4 – orthophosphate, TN – total nitrogen, TP – total phosphorus

The Sørensen similarity index was applied to compare phytoplankton at all sites, using MVSP 3.21 software. The statistical analysis was used to evaluate the coefficients differences in phytoplankton at the sites, simple correlations and multiple regressions were tested with Statistica 10 software. The significance of differences in the number of taxa and abundance of phytoplankton was tested by the U Mann Whitney test. Relationships between the environmental variables and phytoplankton abundance were tested by the Pearson correlation test. Identification of the best predictors of phytoplankton abundance was performed by employing multiple stepwise regressions. The percentage of variation explained by the pattern was based on R^2. Interpretation of the influence of environmental variables on phytoplankton abundance was made on the basis of canonical correspondence analysis (CCA) and was performed using Vegan 1.15.1 (OKSANEN 2009).

Results

The total number of taxa identified at all sites throughout the whole period of study was 61, including 29 Chlorophyta, 17 Bacillariophyceae, 7 Cyanoprokaryota, 4 Euglenophyta and 2 Chrysophyceae. A general tendency was that with increasing distance between the sites the taxonomic similarity decreased, but the differences were minor. Five Bacilliariophyceae taxa: *Aulacoseira* sp., Centrales non-det, *Fragilaria* sp., *Melosira sp*., *Synedra* sp. were characterised by high frequency (80–100%) at all sites (Table 2). Among

Table 2
Taxonomic composition of phytoplankton at sites examined of the lower Odra River in 2009–2011

Specification	Site 1	Site 2	Site 3	Site 4	Site 5	Specification	Site 1	Site 2	Site 3	Site 4	Site 5
Cyanoprokaryota						**Chlorophyta**					
Chroococcus sp.	+	+	+	+	+	*Actinastrum* sp.	+	MF	+	MF	+
Gomphosphaeria sp.	+	+	+		+	*Ankistrodesmus* sp.	+				
Merismopedia sp.	+	MF	+	+	+	*Ankyra* sp.	+	+	+	+	+
Microcystis sp.	MF	MF	MF	MF	MF	*Closterium* sp.	MF	MF	HF	+	+
Oscillatoria sp.	+	+	+	+	+	*Coelastrum* sp.	+	MF	+	+	+
Snowella sp.	+	+	+	+		*Crucigenia* sp.	+	+	+	+	+
Woronichinia sp.		+				*Desmodesmus* sp.	+	MF	MF	HF	MF
Euglenophyta						*Dictyosphaerium* sp.	+	MF	MF	MF	MF
Astasia sp.				+		*Eutetramorus* sp.	+	+	+	+	+
Euglena sp.		+	+	+		*Golenkinia* sp.					+
Phacus sp.	+	+		+	+	*Gonatozygon* sp.	+	+	+		
Trachelomonas sp.	+	+	+	+	+	*Kirchneriella* sp.	+	+			+
Dinophyceae						*Lagerheimia* sp.	+	+		+	+
Ceratium sp.	+	+	+		+	*Micractinium* sp.	+	+	+	+	+
Peridinium sp.	+			+	+	*Monoraphidium* sp.	+	+	+	+	+
Bacillariophyceae						*Oocystis* sp.	MF	MF	MF	MF	MF
Amphora sp.	+	+	+	+	+	*Pandorina* sp.	MF	+	+	MF	MF
Asterionella sp.	+	MF	MF	MF	+	*Pediastrum* sp.	MF	HF	HF	HF	HF
Aulacoseira sp.	HF	HF	HF	HF	HF	*Planktosphaeria* sp.				+	
Caloneis sp.	+	+	+	+	+	*Pteromonas* sp.	+				
Centrales non-det	HF	HF	HF	HF	HF	*Scenedesmus* sp.	HF	HF	HF	HF	HF
Cymatopleura sp.	MF	MF	MF	+	+	*Schroederia* sp.					
Cymbella sp.	+	+	+	+	+	*Selenastrum* sp.	+		+	+	+
Fragilaria sp.	HF	HF	HF	HF	HF	*Spirogyra* sp.			+		
Gomphonema sp.	+	+	+	+	+	*Staurastrum* sp.	+	+	MF	MF	MF
Melosira sp.	HF	HF	HF	HF	HF	*Stichococcus* sp.				+	
Navicula sp.	+	MF	+	MF	+	*Tetraedron* sp.	+				
Nitzschia sp.	+	+	+	+	+	*Tetrastrum* sp.		+		+	+
Pennales-non-det	+	+	+	+	+	*Ulothrix* sp.	+	+		+	+
Pinnularia sp.	+	+	+	+		**Chrysophyceae**					
Pleurosigma sp.	+	+	+	+	+	*Dinobryon* sp.	+			+	+
Surirella sp.	+	+	+	+	+	*Uroglena* sp.				+	
Synedra sp.	HF	HF	HF	HF	MF						

HF – highest frequency (80–100%), MF – intermediate frequency (60–80%). Frequency is the number of occurrences of taxa at sites according to all study periods.

Chlorophyta, one taxon (*Scenedesmus* sp.) showed high frequency and two others (Oocystis sp., Pediastrum sp.) showed intermediate frequency (60–80%). One taxon from Cyanoprokaryota (*Microcystis* sp.) showed intermediate frequency at all sites. The taxonomic similarity between all sites varied from 0.71 to 0.79 (Table 3).

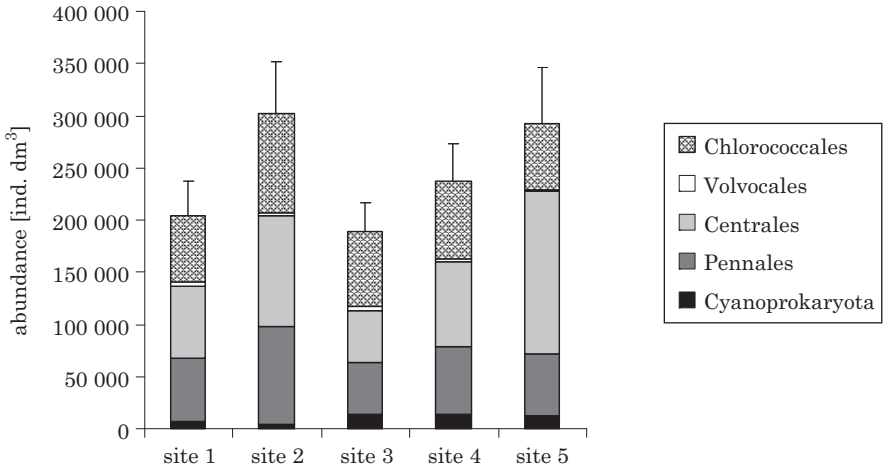
Table 3
Variation of mean taxonomic Sørensen's similarity Index of phytoplankton, between examined sites of the lower Odra River in 2009–2011

Site	1	2	3	4
2	0.76	–	–	–
3	0.74	0.79	–	–
4	0.73	0.75	0.73	–
5	0.72	0.72	0.71	0.78

The taxonomic composition of phytoplankton was similar and no significant differences in the number of taxa at all sites were noted ($P > 0.05$) – Table 2. The highest numbers of taxa were found in the samples from the West Odra River, while the lowest – from the cooling channel. The highest number of taxa represented Chlorophyta and Bacillariophyceae. Chlorophytes were represented on average by 46% of all taxa at all sites, 42% of this number belonged to Chlorococcales, and only 4% to Volvocales. Bacillariophyceae were represented on average by 41% of all taxa noted at all sites, of which 28% belonged to Pennales and 13% to Centrales. Cyanoprokaryota were represented on average by 8% of all taxa at all sites, while the other groups of phytoplankton made about 1% of all taxa.

Similarly as for the number of taxa, no statistically significant differences in abundance of particular groups of phytoplankton were found between the sites ($P > 0.05$); although in the water of the cooling channel (site 3), its abundance was lower than at the other sites (Figure 2). The highest average abundance of phytoplankton was noted at the West Odra River (site 2) and the Odyniec Channel (site 5), while the lowest at the cooling channel (site 1). Relatively low phytoplankton abundance was also noted above the fork of the river (site 1) and in the East Odra River (site 4).

Among all groups of phytoplankton the most abundantly represented were Bacillariophyceae, consisting of Centrales and Pennales. Centrales on average were found in the highest number at site 5 (52.7%) and in the lowest number at site 3 (26.7%). Pennales on average were the most abundant at site 2 (30.6%), while the least at site 5 (20.6%). Abundant were also Chlorophyta, from which Chlorococcales on average were the most abundant at site 3 (37.7%), while the least at site 5 (21.5%), and Volvocales were the most at site 3 (2.1%) and the



Groups which have an abundance of less than 1500 ind. dm^{-3} were not included
Fig. 2. Mean + SD abundance ind. dm^{-3} of Chlorococcales, Volvocales, Centrales, Pennales, and Cyanoprokaryota at sites examined of the lower Odra River

least at site 5 (0.5%). In the phytoplankton the contribution of Cyanoprokaryota varied from 1.5% at site 2 to 7.6% at site 3 of phytoplankton abundance. The contribution of the other groups was less than 1%.
Pearson coefficient calculations indicated many correlations between abiotic factors (Table 1) and phytoplankton groups. Particularly significant were the correlations between temperature and the abundance of Centrales, Volvocales, Chlorococcales ($P < 0.05$) – Table 4.

A significant negative correlation was found between the abundance of Cyanoprokaryota and the dissolved oxygen content ($P < 0.05$). Moreover, a correlation between the conductivity and the abundance of Bacillariophyceae

Table 4
Significant Pearson's correlations between environmental variables and abundance of phytoplankton

Variable	Temp.	O_2	Cond.	N-NH_3	N-NO_3	N-NO_2	P-PO_4	NTOT	PTOT
Cyanoprokaryota	–	-0.415**	–	–	–	–	–	–	0.678***
Euglenophyta	–	–	–	–	–	–	–	-0.309*	–
Dinophyceae	–	0.327*	–	–	–	–	–	–	–
Pennales	–	–	-0.304*	-0.311*	0.530***	–	–	0.447**	-0.323*
Centrales	0.453**	–	0.338*	–	-0.297*	0.328*	–	–	–
Volvocales	0.651***	–	–	–	–	–	0.538***	–	–
Chlorococcales	0.692***	–	0.593***	0.309*	-0.350*	–	0.351*	–	-0.382*

Temp. – temperature, cond. – conductivity, N-NH_3 – ammoniacal nitrogen, N-NO_3 – nitrate nitrogen, N-NO_2 – nitrite nitrogen, P-PO_4 – orthophosphate, NTOT – total nitrogen, PTOT – total phosphorus; Significance *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

(Pennales and Centrales) and Chlorococcales ($P < 0.05$) was noted. The correlation ($P < 0.05$) was also found among the abundance of green algae and total phosphorus, the abundance of Volvocales and orthophosphates, abundance of Pennales and nitrates.

The multiple stepwise regression showed that temperature, ammonium nitrogen contents, nitrates, nitrites, orthophosphates, total nitrogen and total phosphorus were statistically correlated with the abundance of individual taxonomic groups of phytoplankton ($P < 0.05$) – Table 5. From 24% to 68% of variation in abundance of particular taxonomic groups were explained by the analysis. The most significant predictors were: temperature correlated with abundance of Pennales and Volvocales ($P < 0.05$) and the content of total phosphorus correlated with the abundance of Cyanoprokaryota, Dinophyceae, Pennales and Chlorococcales (all $P < 0.05$).

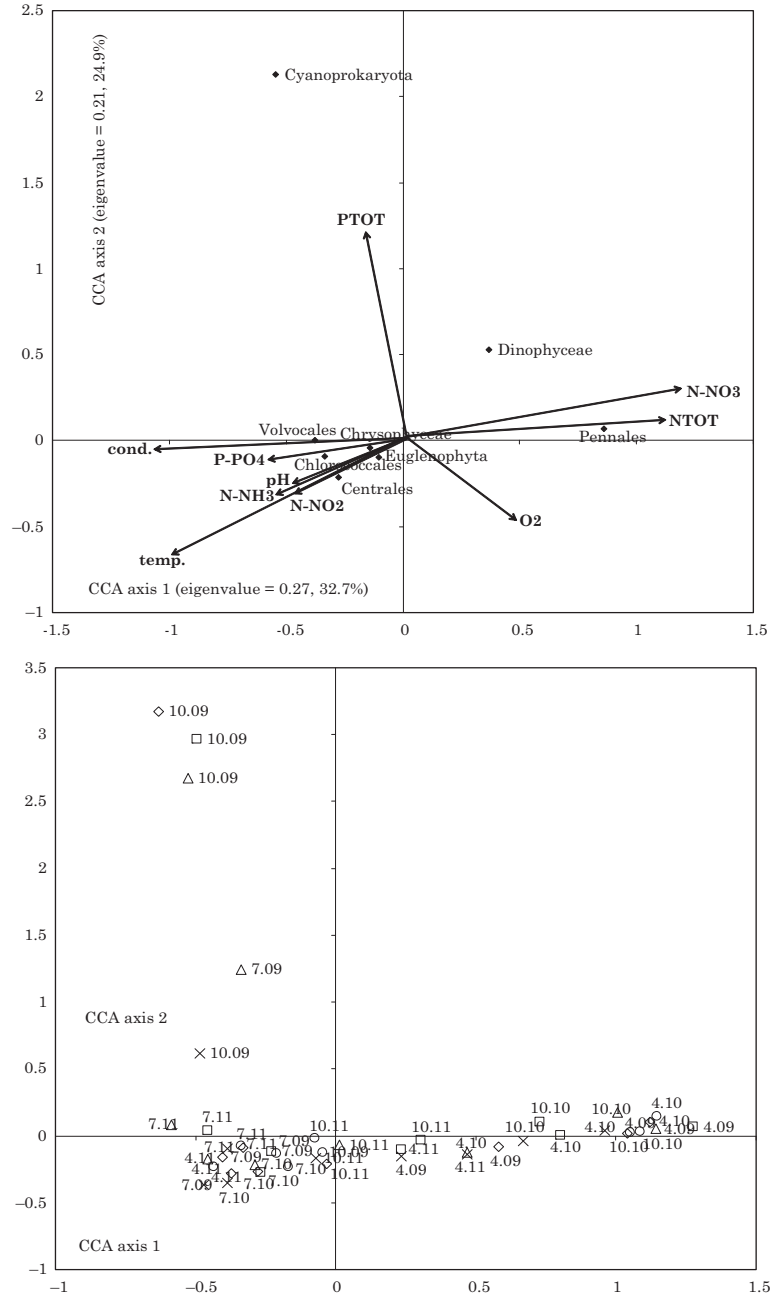
Table 5

Significances of the effects of environmental variables on the abundances of phytoplankton based on multiple regressions (stepwise procedure), with the following dependent variables: abundance of Cyanoprokaryota, Dinophyceae, Pennales, Centrales, Volvocales, and Chlorococcales

Variable	Temp.	N-NH_3	N-NO_3	N-NO_2	P-PO_4	NTOT	PTOT	R^2
Cyanoprokaryota	–	–	–	–	–	–	**	0.61
Dinophyceae	–	–	–	–	–	–	*	0.24
Pennales	**	*	*	–	–	–	***	0.65
Centrales	–	–	–	**	–	–	–	0.44
Volvocales	**	–	*	–	**	*	–	0.68
Chlorococcales	–	–	–	–	–	*	*	0.65

Independent variables taken for analysis were: Temp. – temperature, N-NH_3 – ammoniacal nitrogen, N-NO_3 – nitrate nitrogen, N-NO_2 – nitrite nitrogen, P-PO_4 – orthophosphate, NTOT – total nitrogen, PTOT – total phosphorus; significance *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Canonical correspondence analysis CCA proved a pronounced effect of 10 environmental variables on the abundance of particular taxonomic groups, which explain 57.6% variation in the phytoplankton. The first axis was best correlated with conductivity, the content of total nitrogen, nitrates and temperature (Figure 3). While the second one was best correlated with the content of phosphorus and dissolved oxygen. The separation of results on the CCA axis into two groups according to the seasons indicated a significant influence of the season on the phytoplankton structure and abundance. The first group included the results obtained from April and October, while the second included the results collected from July.



Environmental variables: temp. – temperature, O_2 – dissolved oxygen, pH, Cond – conductivity, $N–NH_3$ – ammonium nitrogen, $N–NO_3$ – nitrate nitrogen, $N–NO_2$ – nitrite nitrogen, $P–PO_4$ – orthophosphate, NTOT – total nitrogen, PTOT – total phosphorus. Sites: 1 – square, 2 – circle, 3 – triangle, 4 – rhombus, 5 – cross. Values indicated as [month, year].

Fig. 3. CCA constrained ordination of taxa and samples from sites in the lower Odra River

Discussion

Taxonomical and quantitative diversity of phytoplankton

The highest number of taxa represented Bacillariophyceae and Chlorophyta, which has also been observed in other large rivers: in the Minho River in Portugal; the Po River in Italy; the Vistula River in Poland, the Moselle River in France (DESCY 1993, DODDS 2006, TAVERNINI at al. 2011, VASCONCELOS and CERQUEIRA 2001).

As follows from the similarity index values, phytoplankton at none of the sites was statistically significantly different in respect of taxonomical compositions. A tendency of decreasing taxonomic similarity with increasing distances between the sites was noted. This is particularly well pronounced with comparison of Site 1 to the other sites. WETZEL (2012) has reported the similarity indices declined with increasing distance between sampling sites. In our study, the actual distances between sites were smaller than those in the study of the above author, but also on a smaller scale this rule was found to hold true. Moreover, in our opinion, the similarity was related to the specific conditions at the sites. The lowest similarity in taxonomic composition was observed between the Odyniec Channel (site 5) and the other sites, which additionally can be attributed to the specific conditions in this channel where the retention time is much longer.

Specific environmental conditions in these channels could stimulate formation of many kinds of niches favoured by plankton species. The highest number of taxa and their relatively high abundance in the West Odra River (site 2) can be explained as a consequence of phytoplankton supply from the channels that join the main bed of the West Odra River and by their development in upstream-reservoirs. Such dam reservoirs change the hydrological and ecological conditions in flowing water and are valuable sources of plankton in rivers (LAIR 2006).

Relatively low phytoplankton abundance at site 1 and site 4 can be explained by the high rate of water flow as described by ALLAN (1998), as increased retention time is known to be favourable for phytoplankton growth.

Interesting results were obtained for the Odyniec Channel (site 5). Thus one expects that higher number of phytoplankton should be observed because as the water flow decreases, the number of euplankton species increases (DEMBOWSKA 2009). However, as our results indicate, the number of phytoplankton in this channel is similar, which can be explained by certain limiting factors such as bivalves filtration and the predatory activity of zooplankton. Furthermore, in this channel, high abundances of *Dreissena polymorpha* (DOMAGAŁA et al., 2004) and *Sinanodonta woodiana* (DOMAGAŁA et al. 2007)

were observed. As CARACO et al. (1997) report, bivalves can significantly restrict the phytoplankton biomass by selective filtration. Moreover, according to GOŁDYN and KOWALCZEWSKA-MADURA (2008), grazing by zooplankton is also an important factor affecting the structure of phytoplankton communities. CZERNIAWSKI et al. (2013) observed in the same time and in the same sites as we sampled the phytoplankton, a high number of zooplankters, in particular crustacean filter feeders reported in the Odyniec Channel (site 5). It seems that although the hydrological conditions were favourable, the phytoplankton composition and abundance in the Odyniec Channel (site 5) were probably determined by the biological conditions.

Impact of physico-chemical variables on phytoplankton abundance

Eutrophic rivers carry a high concentration of nutrients needed for phytoplankton growth (TAVERNINI et al. 2011). However, as found by REYNOLDS (1994) and as indicated by the results of present study, there is a positive correlation between the content of inorganic nutrients and the abundance of phytoplankton. This indicates that the content of nutrients has proved to be a limiting factor for phytoplankton growth. The correlations between groups of phytoplankton and inorganic nutrients seem to be caused by two factors: seasonal changes in the content of phosphorus and nitrogen (related to run-off from the fields) and different hydrological conditions at the studied sites. Hitherto hydrological variables were not measured by us, but according to literature these variables are the main factors that affect the plankton development in rivers (ALLAN 1998).

The content of both N and P (in organic and inorganic forms) can be important determinants of autotrophic and heterotrophic activity in rivers and streams as established by DODDS (2006). A positive correlation was found between the total phosphorus content and the abundance of Cyanoprokaryota, which was the most significant in the cooling channel (site 3), where elevated temperature accelerated eutrophication which was unambiguous in the decreasing of dissolved oxygen concentration and favoured the growth of Cyanoprokaryota. Correlations can be also found among the abundance of each Cyanoprokaryota, Dinophyceae, Pennales, Centrales, Volvocales, Chlorococcales and nutrients, which supports the thesis (TAVERNINI 2011) about the positive influence of nitrogen and phosphorus on the phytoplankton composition.

Water discharge itself produces changes in the physical and chemical condition, thus affecting phytoplankton assemblages (DESCY 1993). This can be seen in low-gradient rivers with long retention times. As indicated in

(REYNOLDS 1994), the micro-algal abundance is inversely correlated with the discharge rate and turbidity, and positively correlated with the content of nutrients. We have also observed such relationships; however, the effect of discharge rate and turbidity must be further investigated. The results of CCA showed that the factors which explain the differences in the phytoplankton abundance and composition at the sites studied with a rather high probability. The most important of these factors are the temperature and the content of nutrients, which is in full agreement with the results of correlation test and multiple stepwise regressions. Furthermore, the seasons also influence the content of nutrients, e.g. P and N. The separation of results obtained in April, July and October in CCA pattern indicates the most important impact on the phytoplankton composition and the abundance in the lower Odra River due to seasonal changes. Other authors have also reported more abundant structures of phytoplankton in summer months (DESCY 1993, GOŁDYN and KOWALCZEWSKA-MADURA 2008).

Impact of cooling water

Many authors have been interested in the effect of cooling water from power plants on phytoplankton (JORDAN et al. 1983, MARTINEZ-ARROYO et al. 2000, POORNIMA 2006, RAYMOND and RAYMOND 1969, TYSZKA-MACKIEWICZ 1983, ZARGAR and GHOSH 2006). It seems that such ecosystems are not homogenous and host many factors limiting phytoplankton development. Low phytoplankton abundance in cooling channels has been observed by many authors: MULFORD (1974) Patuxent Estuary, Maryland; GOLDMAN and QUINBY (1979) Cape Cod and Montaup; BRIAND (1975) San Gabriel River, California. Although no statistically significant differences were noted in the mean abundances of phytoplankton between the studied sites, the lowest number of taxa and the lowest average phytoplankton abundance were noted in the cooling channel (site 3). It could be expected that probably slow water current in the cooling channel had a positive effect on phytoplankton growth, but there could be many factors restricting it, such as:

1) Mechanical stress (LANGFORD 1990).

2) Predation by bivalve molluscs, (e.g. *Dreissena polymorpha, Corbicula fluminea* (ŁABĘCKA et al. 2005) feeding on phytoplankton as discussed earlier (CARACO et al. 1997, COHEN et al. 1984).

3) And the lethal effect of elevated temperatures (CRIPPEN et al. 1978, FLEMER and SHERK 1977, WARINNER and BREHMER 1966, WELCH and WARD 1978). However, the effect of an elevated temperature seems difficult to explain as water temperatures reaching 30°C are observed in the cooling channel only

periodically in the summer. According to MARTINEZ-ARROYO et al. (2000) water temperatures above 30°C can lead to a decrease in the photosynthetic abilities in phytoplankton. On the basis of our results and those of other authors, it seems that the power plant cooling system restricts the abundance of phytoplankton, but has no significant effect on its taxonomic composition. Seasonal changes were crucial to the production of phytoplankton composition and the effects of heated water were minor.

Conclusion

The most pronounced differences were revealed in the phytoplankton abundance, which, according to the statements of cited authors, is probably related to the time of water retention, the washing out of plankters from slack water, predation by mollusks and zooplankton. Among the factors subjected to statistical analysis, the strongest correlation was between the phytoplankton abundance and inorganic nutrients, the next most important factor was the temperature. The effect of temperature seems to be related to seasonality temperature rather than to heated water from power plant. The phytoplankton in the cooling channel had a taxonomic structure similar to those at the other sites, but it was less abundant. The cooling water from the power plant seems to accelerate eutrophication in discharge but have no significant impact on phytoplankton composition downstream in the Odra River.

Translated by MARIA SPYCHALSKA

Accepted for print 1.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 153–160, Y. 2014

A NOVEL POLYMORPHISM WITHIN INTRON B OF GROWTH HORMONE GENE (*GH2*) OF THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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K e y w o r d s: rainbow trout, *Oncorhynchus mykiss,* growth hormone gene, PCR-RFLP.

A b s t r a c t

Nucleotide composition of both growth hormone variants of rainbow trout (*Oncorhynchus mykiss*) has been strongly preserved evolutionally what might suggest that any change within these sequences can have an influence on the functioning of the somatotropic axis. A 121 bp fragment that contained nearly the entire B intron was amplified by the polymerase chain reaction. PCR products were bidirectionally sequenced. PCR products were digested by TaiI according to manufacturer's instructions and resulting DNA was subjected to electrophoresis. An analysis of the gene fragment for growth hormone 2 showed the presence of SNP, easily identifiable by means of digestion with *Tai*I restriction enzyme. Statistical analysis confirmed that homozygous GH^{BB} fish were the longest (31.77 cm) and the heaviest (404.70 g) and were statistically significantly different ($P \leq 0.05$) from heterozygous GH^{AB} fish. Mean length of GH^{AA} homozygous fish was insignificantly lower (30.06 cm) with mean body weight of 339.12 g than homozygotes GH^{BB}.

CHARAKTERYSTKA POLIMORFIZMU W SEKWENCJI INTRONU B GENU HORMONU WZROSTU (*GH2*) PSTRĄGA TĘCZOWEGO, *ONCORHYNCHUS MYKISS*

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Abstrakt

Sekwencja nukleotydowa obu wariantów genu hormonu wzrostu pstrąga tęczowego (*Oncorhynchus mykiss*) jest silnie zakonserwowana ewolucyjnie. Każde nowo powstałe miejsce polimorficzne może mieć więc wpływ na funkcjonowanie osi somatotropowej. Badano niepełną sekwencję intronu B genu hormonu wzrostu typu 2 o długości 121 pz, uzyskaną po przeprowadzeniu dwukierunkowej reakcji sekwencjonowania. Otrzymane produkty PCR poddano analizie z wykorzystaniem endonukleazy *Tai*I, a wynik tej reakcji zobrazowano poprzez rozdział otrzymanych fragmentów restrykcyjnych w żelu agarozowym. Podczas analiz wykazano obecność mutacji punktowej, którą można łatwo zidentyfikować poprzez trawienie sekwencji nukleotydowej intronu B enzymem restrykcyjnym *Tai*I. W analizie statystycznej otrzymanych danych potwierdzono, że homozygotyczne GH^{BB} pstrągi o średniej długości 31,77 cm oraz masie 404,70 g różniły się statystycznie istotnie ($P \leq 0,05$) od osobników heterozygotycznych GH^{AB}. Średnia długość (30,06 cm) oraz masa (339,12 g) homozygotycznych GH^{AA} ryb były nieistotnie niższe od analogicznych parametrów osobników z genotypem GH^{BB}. Scharakteryzowany polimorfizm ma istotny statystycznie wpływ na tempo wzrostu badanych osobników pstrąga tęczowego.

Introduction

The rainbow trout (*Oncorhynchus mykiss*) plays an important role in Polish and world aquaculture. In 2010, Polish aquaculture production of this species amounted to approximately 13 000 tonnes and came second after the carp (*Cyprinus carpio*) with 15 400 tonnes (FAO 2010). In order to speed up process of trout growth a complex mechanism of growth regulation was analysed, especially taking into account the somatotropic axis. A major element of the axis is the growth hormone, whose synthesis and secretion take place in somatotropic cells of the pituitary gland under control of *Pit-1* factor (LEFEVRE et al. 1987, BOLLIET et al. 2001). An analysis conducted on *O. mykiss* lead to a discovery of the presence of two *GH* genes: *GH1* and *GH2*, resulting from genome-doubling event that occurred 25-100 million years ago (ALLENDORF and THORGAARD 1984). *GH1* and *GH2* mRNA consist of 630 nucleotides and encode 210 amino acid residues. Both forms differ by 22 nucleotides in the protein-coding region and their synthesis depend on sex, age and density of the fish. Level of mRNA *GH2* was reported lower than *GH1*, specifically in pituitary glands of 10-day-old fry and 2-year-old females (AGELLON et al. 1988, YANG et al. 1997). Moreover, YANG with co-authors (1997) revealed within 5' flanking regions, exons and introns of *GH1* and *GH2* sequences relating to the cAMP-response elements, thyroid hormone-response elements, retinoic acid-response elements, estrogen-response element (only in *GH1*), and glucocorticoid-response elements. Nucleotide sequences of both growth hormone variants have been strongly preserved in the course of evolution which might suggest that any change within these sequences can have an influence on the functioning of both the somatotropic axis and growth performance (RENTIER-DELRUE et al. 1989).

Selection of fish based on various genetic markers has resulted in a faster growth rate of the trout (O'MALLEY et al. 2003, DREW et al. 2007). One from the interesting markers are SNP's (single nucleotide polymorphism) that may occur in both coding and non-coding regions (BLACK 2003, DE-SANTIS, JERRY 2007, HE et al. 2012). Scientific literature provide examples in which point mutations located within *GH* sequence influence some productive traits of fowls, goats, cows or less frequently fish (LAGZIEL et al. 1999, MARQUES et al. 2003, LEI et al. 2007, AMINAFSHAR and REZA 2012, NI et al. 2013). Despite the fact that most SNP's occur in introns, these non-coding regions of gene may influence processes of transcription, translation or expression, which in turn might affect growth performance (NI et al. 2013). None of the point mutations found in the less abundant *GH2* (comparing to *GH1*) were associated with growth performance of rainbow trout so far. Therefore, the purpose of this study was to analyse the non-coding part of *GH2* sequence for polymorphism and to assess if the found polymorphism might be associated with a higher growth rate.

Materials and Methods

A total of 97 trout individuals were randomly caught alive during spring time from concrete tank of the fish farm Mołstowo which is constantly supplied with water form River Mołstowa. Fish had the same culture conditions (feed, temperature) and were at the same age. Total length was measured using callipers with 0.01 mm accuracy and weight was assessed using weighting scale with 1g accuracy. Before the fish was released a small piece of caudal fin from each of the trout was dissected and placed in 1.5 ml Safe-Lock micro test tubes (Eppendorf Inc.). DNA extraction was carried out according to standard phenol chloroform extraction method. Purity and concentration of DNA extracts were analysed on a 1% agarose gel and Nanodrop ND-1000 (Thermo Fisher Scientific Inc.) spectrophotometer, then stored in -20°C until PCR (polymerase chain reaction) assays.

According to the sequence of the rainbow trout *GH*2 gene (GenBank acc. code DQ294400) a pair of specific primer sequences was designed using Primer3 program (Table 1). Sequence submitted into GenBank was obtained during our earlier (unpublished) studies. Primers that enabled amplification and sequencing of 381 bp sequence had to be redesigned and as a next step in the presented study authors designed and used *GH*2F and *GH*2R. Hence, length differences between these sequences. A 121-base pair (bp) fragment that contained nearly the entire B intron was amplified by the PCR. *GH* intron sequences had often been used to infer sub-familial phylogenetic relationships

amongst salmonids (OAKLEY and PHILLIPS 1999). Additionally, KIRKPATRICK (1992) described that point mutation in pig intron B of *GH* gene is correlated with important performance traits. The PCR reaction for each sample contained 90 ng of genomic DNA, 10 pmol of each primer, 2 μl 10x PCR Buffer with $(NH_4)_2SO_4$ (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 1.2 μl 25 mM $MgCl_2$, 2 μl dNTP mix and 0.5 units of Taq-polymerase (MBI Fermentas), amounting to total volume of 20 μl. The PCR reactions were performed in a thermal cycler (Perkin Elmer) programmed for initial denaturation in 5 mins at 94°C followed by 35 cycles of 45 secs at 94°C, 1 min at 59°C and 1 min at 72°C, and a final extension over 5 mins at 72°C. After the amplification, PCR products were subjected to electrophoresis on 2% argarose gel. Ten PCR products of each variant were bidirectionally sequenced (IBB PAN, Warsaw, Poland) and analysed with the aid of Chromas (Technely-sium Pty Ltd, Tewantin, Australia) and BioEdit software (HALL 1999). Additionally, all obtained sequences were analysed using the on-line Webcutter 2.0 program to select the restriction enzyme. PCR products were digested by *Tai*I (Fermentas) according to manufacturer's instructions and resulting DNA was subjected to electrophoresis on 2% argarose gel. Significance of the observed differences was analysed based on Duncan's multiple range test.

Table 1

Primers used to amplify the analysed region of *GH*2 gene of the rainbow trout

Name	Sequence	Position of the amplified fragment
*GH*2F	5' TTGAATCTTCTTTTGACACAGCA 3'	110–230*
*GH*2R	5' CAAAATCACAAAGACGGGAGA 3'	

* according to GenBank access code DQ294400

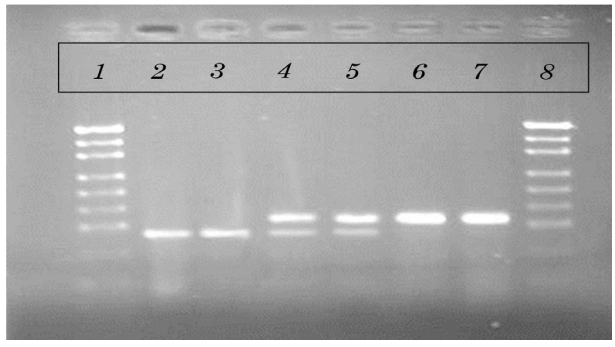
Results

Restriction site predicted using Webcutter 2.0 and verified by digestion proved that *Tai*I recognized the mutation site T → C (recognition sequence acgt↓). The following DNA restriction fragments were obtained for the *rtGH2*/*Tai*I polymorphism (Figure 1):

– 90 and 31 bp for the AA genotype (C at the position 138 (GenBank access code DQ294400) – 2 and 3 lanes;

– 121, 90 and 31 bp for the AB genotype (heterozygote) – 4 and 5 lanes;

– 121 bp for the BB genotype (T at the position 138 (GenBank access code DQ294400); no digestion) – 6 and 7 lanes.



1 and *8* lanes – DNA ladder pUC 19/*Tai* I (9 fragments in bp – 501, 404, 331, 242, 190, 147, 110, 67, 34)
Fig. 1. Electrophoretic pattern of polymorphic GH2 gene of the rainbow trout

Sequence analysis revealed in the analysed amplicons only one site where base pair was T or C, depending on the genotype of the trout. A total of 97 individuals were genotyped (Table 2).

Table 2

Frequencies of genotypes and alleles of the rainbow trout *GH*2 gene

Rainbow trout GH2 gene	rtGH2/TaiI genotype			All	Frequencies of alleles	
	AA	AB	BB		A	B
n	25	48	24	97	0.5052	0.4948
Frequencies of genotypes	0.2577	0.4948	0.2475	1.000		
Length [cm]	30.06 ± 3.91	29.27^a ± 3.92	31.77^a ± 4.13	–	–	–
Weight [g]	399.12 ± 105.41	322.25^a ± 133.36	404.70^a ± 145.08	–	–	–

a – $P \leq 0.05$

Statistical analysis confirmed that homozygous GH^{BB} fish were the longest (31.77 cm) and the heaviest (404.70 g) and were statistically significantly different ($P \leq 0.05$) from heterozygous GH^{AB} fish (322,25 g and 29,27 cm). Mean length (30.06 cm) and body weight (339.12 g) of GH^{AA} homozygous fish was insignificantly lower comparing to homozygous GH^{BB} trout. Mean length and weight of the analysed individuals are given in the Table 2.

Discussion

On the basis of the above data it is obvious that the genotype GH^{BB} had the biggest influence on growth rate of fish in the analysed stock. In spite of that, this kind of mutation occurring in the intron sequence might have a consider-

able influence on the growth and development of the rainbow trout. It is supposed that the location of this mutation might have an influence on mRNA *GH2* splicing and proper functions of growth hormone protein. Literature of the subject has provided examples of intron point mutations that had influence on fish weight gain, e.g. point mutation found within the third intron of growth hormone gene of the Atlantic salmon (*Salmo salar*), which turned out to have substantial influence on the growth rate (GROSS 1999). In their study, TAO and BOULDING (2003) examined the pituitary adenylate cyclase activating polypeptide gene (PACAP) and growth hormone-releasing hormone (GHRH) gene, which had a common mRNA promoter. The researchers concluded that point mutation within the fourth intron conditioned an alternative splicing and determined the presence of two mRNA forms: the shorter PACAP and the longer GHRH, in brains and intestines of the arctic charr (*Salvelinus alpinus*). A practical aspect of the study was the description of a genetic marker which, depending on whether it was G or C, affected growth rate of the species in a manner which was statistically significant. There are also point mutations which do not refer directly to GH structure but have a significant influence on fish growth. A mutation within the intron of laminin-$\alpha 2$ (LAMA 2) gene disturbs the process of splicing and thus non-functional protein is produced. Such a mutation results in the detachment of myofibers, damaged myosepta and growth defects in the brain and eye of the mutant fish, which adds up to congenital muscular dystrophy (GUPTA et al. 2012). In another analysis, SÁNCHEZ-RAMOS et al. (2012) showed a significant connection between MSTN-1 gene polymorphism and growth traits for the gilthead seabream (*Sparus aurata*).

The type of mutation presented in this paper has usually been regarded as unimportant and unlikely to have any possible influence on fish growth rate. However, the novel mutation found in this study in the intron sequence of growth hormone gene 2 has a substantial influence on the length and weight of the rainbow trout. Statistical analysis has confirmed that homozygous GH^{BB} fish were the longest and the heaviest among all fish in the tested group. The analysis discussed here will be continued to find additional polymorphic sites which will be tested in more numerous groups of individuals. A prepared group of genetic markers supported by statistical analysis might be applied to marker assisted selection (MAS) programs aimed at growth rate improvement.

Conclusions

1. Based obtained results it is highly probable that the genotype have influence on lengths and weights of rainbow trout in the analysed tank.

2. Mutation occurring in the intron B sequence of *GH* have a considerable influence on the growth rate of the rainbow trout.

3. Statistical analysis confirmed that fish with GH^{BB} genotype had the best predisposition for growth among all fish in the analysed group.

Translated by Remigiusz Panicz

Accepted for print 1.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 161–175, Y. 2014

GENETIC VARIABILITY OF *SALMO TRUTTA* L. SPECIES FROM THE CATCHMENT AREAS OF THE DRAWA AND REGA RIVERS EVALUATED USING RAPD AND SSR MARKERS*

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K e y w o r d s: genetic polymorphism, microsatellite sequences, RAPD, brown trout, sea trout.

A b s t r a c t

The knowledge of the genetic variability and structure of *Salmo trutta* population is needed for effective protection of the species and rational management of the resources. A number of marker systems have been introduced to evaluate the genetic variability of trout populations. Among them, the most often used are the RAPD and SSR markers. Both marker systems are classified as type II markers (O'BRIEN 1991, LERCETEAU-KÖHLER and WEISS 2006).

In this study, the genetic variability of the *Salmo trutta* m. *fario* and *Salmo trutta* m. *trutta* populations from the Rega river, and the three watercourses Sitna, Słopica and Bagnica of the Drawa river catchment area, were analysed. One stream, the Chojnówka (located outside the catchments of the above streams), was used as an extra study area.

Based on two marker systems, different results were obtained. In the case of RAPD analysis, all loci were polymorphic in all populations. The use of these marker systems permitted the construction of UPGMA similarity trees. The trees revealed a division of the analysed populations into two groups: one group from the Słopica river and the other group from the remaining watercourses. In the second similarity group, two subgroups can be distinguished: one comprising the population of the sea trout from the Rega river and that of the brown trout from the Sitna river (60.7%), and the other consisting of the parr trout populations from the Chojnówka, Bagnica and Sitna (50.3–79.4%). Between the analysed populations, 100% polymorphism was found. The results indicate a high genetic variability of the studied populations. In the case of SSR analysis, 9 microsatellite loci isolated from five trout populations were described. The number of alleles at these loci ranged from 1 to 5 with an average of 2.8 alleles per locus. The expected heterozygosity ranged from 0.07 to 0.66, with an average of 0.35. The results indicate high genetic variation of the populations studied.

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* This study was partially financed by the European Union Sectoral Operational Programme *Sustainable development of the fisheries sector and coastal fishing areas 2007–2013*, no. 00001-61724-OR1600004/10

OCENA ZMIENNOŚCI GENETYCZNEJ GATUNKÓW *SALMO TRUTTA* L. Z CIEKÓW ZLEWNI DRAWY I REGI ZA POMOCĄ MARKERÓW RAPD I SSR

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S ł o w a k l u c z o w e: polimorfizm genetyczny, sekwencje mikrosatelitarne, RAPD, pstrąg potokowy, troć wędrowna.

A b s t r a k t

W pracy badano zmienność między populacjami *Salmo trutta* m. *fario* i *Salmo trutta* m. *trutta* pochodzącymi z Regi oraz trzech cieków zlewni Drawy: Sitnej, Słopicy i Bagnicy na podstawie analizy RAPD i SSR. Do analizy dodano jedną populację – Chojnówkę zlokalizowaną poza zlewnią Drawy.

Na podstawie dwóch systemów markerowych otrzymano zróżnicowane wyniki. W przypadku analizy RAPD wszystkie loci były polimorficzne we wszystkich populacjach. Drzewo UPGMA przedstawia podział analizowanych populacji na dwie grupy: do pierwszej należy jedynie populacja Słopicy, a do drugiej wszystkie pozostałe. W pracy stwierdzono także występowanie 100% polimorfizmu między analizowanymi populacjami. W przypadku analizy SSR badano 9 loci mikrosatelitarnych w 5 populacjach. Drzewo UPGMA na podstawie tego systemu markerowego przedstawia podział badanych populacji na dwie grupy podobieństwa: jedną złożoną z populacji z Regi i drugą, do której należą pozostałe populacje. Populacja dorosłych oraz troci w stadium parr z Sitnej tworzą odrębną podgrupę podobieństwa.

Otrzymane wyniki wskazują na bardzo duże zróżnicowanie genetyczne badanych ryb.

Introduction

The data on the genetic structure of fish species or populations are needed for the identification of taxa, implementation of rearing programmes and preservation of the genetic variability of the species. The evaluation of genetic variability in fish is performed based on morphological criteria (ANDERSON et al. 1993), allozymes (CAGIGAS et al. 1999, MITH et al. 1997), RFLP (HALLERMAN and BECKMANN 1988), minisatellite and microsatellite sequences (TAGGART and FERGUSON 1990), and RAPD markers (DERGAM et al. 1998, LIU et al. 1999, NADIG et al. 1998).

The preservation of biodiversity at the genetic level in water ecosystems, including watercourses, has been an object of concern for many years in Poland and all over the world. A particular object of concern in this aspect were salmonids (ACHORD et al. 2007, CZERNIAWSKI et al. 2010, DOMAGAŁA and BARTEL 1997, 1999, MCNEIL 1991), whose population is seriously threatened because of high mortality rate of the larvae introduced, problems with migra-

tion, poaching or irresponsible stocking with individuals from different populations (BARTEL 2001, BROWN 2002, JONSSON and JONSSON 2006, SALVANES et al. 2001). The latter procedure can lead to the breaking up of the genetic continuity of indigenous fish and, finally, to the population weakening (SKUZA et al. 2009). Therefore, evaluation of the similarity of local fish populations at all levels of biodiversity, including the genetic level, is needed.

The great geographical diversity of the brown trout was described first on the basis of morphological data, and then on the basis of isoenzyme analyses (OSINOV 1984, PRESA et al. 1994, GIUFFRA et al. 1996) and mitochondrial DNA analyses (BERNATCHEZ et al. 1992, GARCIA-MARIN and PLA 1996). Recently, microsatellite sequences have been widely studied for the evaluation of genomic changes in Salmonidae (FRITZNER et al. 2001, LARGIADER and SCHOLL 1996). Random amplification of polymorphic DNA fragments, the RAPD method, has been used for the preliminary assessment of genetic diversity within a single species or population (ALI et al. 2004, BIELAWSKI and PUMO 1997, CACCONE and 1997, DERGAM and 1998, LIU et al. 1999, NADIG et al. 1998, FOO et al. 1995, POSTELTHWAIT et al. 1994). RAPD markers are inherited as Mendelian markers in a dominant manner, and RAPD band is produced by either homozygotes or heterozygotes, and though band intensity may differ, variations in PCR efficiency make scoring of band intensities problematic. It is difficult to determine whether the bands represent different loci or alternative alleles of a single locus, thus the number of investigated loci can be assessed erroneously. The polymorphism obtained by means of this method is a result of mutations within the sequences complementary to the primer sequences (BRYLIŃSKA 2000). The main advantage of the PCR-RAPD method is the fact that it uses a universal set of primers. In view of the practically infinite number of different ten-nucleotide primer sequences, this method is particularly useful in the search for markers of important characteristics, in the construction of genetic maps and in the evaluation of the genetic similarity of taxa (ALI et al. 2004). Microsatellite DNA sequences are short tandem repetitions of 1–5 nucleotides occurring in the genomes of eukaryotic organisms with a high frequency and a relatively uniform distribution every 6–10 kbp (BECKMANN and WEBER 1992). The polymorphism of microsatellite markers and the fact that the sequences flanking them are specific for certain DNA regions are useful diagnostic characteristics for genome mapping, coupling analysis, population genetics, as well as phylogenetic and evolutionary studies.

Earlier isoenzyme and cytogenetic studies have revealed the small genetic diversity of the sea trout populations from, e.g., the Rega river, related to the past mixing of these populations (ŁUCZYNSKI and BARTEL 1997, ŁUCZYNSKI et al. 2000). Analyses based on microsatellite sequences have revealed great diversity of the fish populations from Polish rivers located close to the Baltic

Sea (WAS and WENNE 2002). The genetic variability of *Salmo trutta* from the Rega river was compared with that of the parr trout and the adult brown trout from small watercourses in the Drawa river catchment area: Sitna, Słopica and Bagnica, and from the Chojnówka river (extra stream). This comparison was made in view of the supposition that these watercourses had been stocked with the stocking material of trout (*Salmo trutta*) from the Rega river. The analysis was based on microsatellite sequences and RAPD markers.

Experimental procedure

Research subject. For the study, we used adults individuals of the brown trout (*Salmo trutta* m. *fario*) from the Sitna and Słopica streams located in the buffer zone of Drawieński National Park (DPN) and stocked parr individuals of the sea trout (*Salmo trutta* m. *trutta*) from the Sitna and Bagnica streams (also located in the buffer zone of DPN), as well as the Chojnówka used as an extra stream flowing in the Beech Forest within the administrative boundaries of Szczecin City (Figure 1). The above fish were compared with spawning individuals of the sea trout from the Rega river. The trout from the Słopica stream are a local herd, while the Bagnica and Sitna streams, many years ago, were stocked with the brown trout derived from individuals from the Rega river. The Chojnówka stream in previous years were stocked with the brown trout coming from the Rega, which now is the settled form and reproduces in the watercourse. The Rega river is the major river of Western Pomerania inhabited by the trout. The stock material was derived from the spawners from the river. All the fish were captured in autumn 2009 with electric fish gear. A sample from each population consisted of 10 fish, except the Chojnówka from which 4 fish were examined. The *Salmo trutta* species belongs to salmonids. It is divided into either purely freshwater populations: *Salmo trutta* morpha *fario* and *S. trutta* morpha *lacustris*, or the anadromous population of *S. trutta* morpha *trutta*, known as the sea trout. Each population has the same initial development stages in the first year of life: newly hatched larvae – alevins, fry and parr.

Isolation of genomic DNA. The DNA was extracted from 0.2 g muscle tissues of particular individuals of investigated fish population. The tissues was put in 1.5 ml microtubes and 1 ml extraction buffer was added (100 mM Tris-HCl, 200 mM NaCl, 0.2% SDS, 5 mM EDTA and 100 μg/ml proteinase K). Mixture was incubated for 12 h at 55°C in a heating block and centrifuged at 6000 rpm for 15 min. Supernatant was transferred to a 1.5 ml microcentrifuge tube and 700 μl of isopropanol was added. The preparations were spined at 6000 rpm for 15 min. After precipitation the DNA was washed with 400 μl 70%

ethanol. The phases were separated by centrifugation at 6000 rpm for 5 min. Supernatant was thrown out and the pellet was allowed to dry. The pellet was dissolved in 20 µl TE buffer and stored at -20°C. Quantify and purity of DNA was analysed by spectrophotometer SmartSpec™Plus (BioRad, USA).

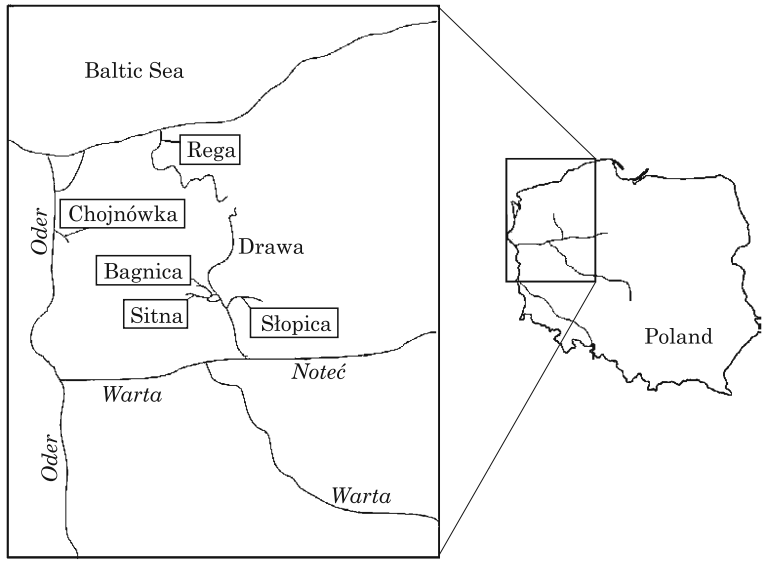


Fig. 1. Study area. The names of rivers from which the fish were sampled are in the frames

PCR-RAPD analysis. All DNA preparations extracted from muscle tissues used in the reaction were diluted to 120 ng μl^{-1}. Among 10 RAPD primers (10 bases-long) tested only 5 gave good quality results (C-02 GTGAGGCGTC, C-04 CCGCATCTAC, C-06 GAACGGACTC, C-11 AAAGCTGCGG, C13 AAGCCTCGTC). The reaction volume of 25 µl was composed of 120 ng μl^{-1} of DNA, 1xGreen GoTaq™ Reaction Buffer (Promega, USA), 2 mM $MgCl_2$, 2 mM dNTP (Fermentas, Lithuania), 0.2 mM primer (Operon Technologies, USA), 0.2 U GoTaq™ DNA Polymerase (Promega, USA). The reaction profile was as follows: a) preliminary denaturation at 95°C for 2 min; b) 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, elongation at 72°C for 2 min; c) final elongation at 72°C for 10 min. Amplification was done by Thermal Cycler MJ Mini (BioRad, USA). Reaction products were separated by electrophoresis in 1.5% agarose gels in 1 × TBE at 80 V. Gels were stained with ethidium bromide (0.35 µg ml^{-1}), and documented in GelDoc XR apparatus. The sizes of amplification products were determinated by comparison with molecular weight standard GeneRuler (Fermentas, Lithuania).

Table 1

Primer microsatellite sequences used in this study

Primer	Repeat motif	Primer sequences	References
Str15INRA	CT	5'-TGCAGGCAGACGGATCAGGC-3 5'-AATCCTCTACGTAAGGGATTTGC-3'	(Estoup et al. 1993)
Str60INRA	GT	5'-CGGTGTGCTTGTCAGGTTTC-3' 5'-GTCAAGTCAGCAAGCCTCAC-3'	(Estoup et al. 1993)
Str73INRA	GT	5'-CCTGGAGATCCTCCAGCAGGA-3' 5'-CTATTCTGCTTGTAACTAGACCTA-3'	(Estoup et al. 1993)
Str79INRA	GT	5'-GGAAGGGGGGTGTATCAGC-3' 5'-GGGATTTGGCCTGTATCCG-3'	(Presa and Guyomard 1996)
Str85INRA	CT	5'-GGAAGGAAGGGAGAAAGGT-3' 5'-GGAAAATCAATACTAACAA-3'	(Presa and Guyomard 1996)
Ssa197	GTGA (+GT)	5'-GGGTTGAGTAGGGAGGCTTG-3' 5'-TGGCAGGGATTTGACATAAC-3'	(O'Reilly et al. 1996)
Str543INRA	CT	5'-ATTCTTCGGCTTTCTCTTGC-3' 5'-ATCTGGTCAGTTTCTTTATG-3'	(Presa and Guyomard 1996)
Str591INRA	CT	5'-CTGGTGGCAGGATTTGA-3' 5'-CACTGTCTTTCGTTCTT-3'	(Presa and Guyomard 1996)
BS131	GT	5'-CACATCATGTTACTGCTCC-3' 5'-CAGCCTAATTCTGAATGAG-3'	(O'Reilly et al. 1996)
T3-13	GT	5'-CCAGTTAGGGTTCATTGTCC-3' 5'-CGTTACACCTCTCAACAGATG-3'	(Estoup et al. 1998)
Str43INRA	GT	5'-GTTGTGGGCTGAGTAATTGG-3' 5'-CTCCACATGCATCTTACTAACC-3'	(Estoup et al. 1998)
Strutta 58	GT	5'-AACAATGACTTTCTCTGAC-3 5'-AAGACTTGAAGGACGAC-3'	(Poteaux 1995)
Strutta 12	GT	5'-AATCTCAAATCGATCAGAAG-3' 5'-AGCTATTTCAGACATCACC-3'	(Poteau 1995)
Ssa 171	GTGA(+GTP)	5'-TTATTATCCAAAGGGGTCAAAA-3' 5'-GAGGTCGCTGGGGTTTACTAT-3'	(O'Reilly et al. 1996)
Ssa 85	GT	5'-AGGTGGGTCCTCCAAGCTAC-3' 5'-ACCCGCTCCTCACTTAATC-3'	(O'Reilly et al. 1996)
OmyFgt1TUF	GT	5'-AGATTTACCCAGCCAGGTAG-3' 5'-CATAGTCTGAACAGGGACAG-3'	(Sakamoto et al. 1994)
SsoSL417	GT	5'-TTGTTCAGTGTATATGTGTCCCAT-3' 5'-GATCTTCACTGCCACCTTATGACC-3'	(Slettan et al. 1995)
SsoSL438	GT	5'-GACAACACACAACCAAGGCAC-3' 5'-TTATGCTAGGTCTTTATGCATTGT-3'	(Slettan 1995)

PCR-SSR analysis. Sixteen microsatellites sequences were studied (Table 1), including one tetra-necleotide loci, and of those successfully amplifying, nine were selected for analysis: Str85INRA Ssa197 Str60INRA, Str43INRA, Strutta 12, BS131, Strutta 58, Ssa85 and SsoSL417. Each amplification reactions were carried out in a final volume of 20 μl containing 120 ng μl^{-1} of DNA, 1x PCR buffer (Promega, USA), 2.5 mM MgCl2, 0.2 mM

of dNTPs (Fermentas, Lithuania), 0.5 mM primer (IBB PAN, Poland) and 0.2U GoTaq™ DNA Polymerase (Promega, USA). DNA amplifications for individual primers were performed in a Thermal Cycler MJ Mini (BioRad, USA) with an initial denaturation of 4 min at 95°C, followed by 30 cycles of denaturation DNA for 30 s at 95°C, annealing at 55°C (Str85INRA) or 60°C (Ssa197) for 45 s, elongation at 72°C for 2.5 min and a final 5 min extension at 72°C. PCR products were analysed for length variation on 6% polyacrylamide gels containing 7 M urea and documented in GelDoc XR apparatus. To check consistency of the results from gels, series of rerun sessions were made.

Statistical analysis

The results were documented in the Bio-Rad gel documentation system and analysed in Quantity-One® software (BioRad, USA).

RAPD analysis. The products of amplification that were repeatable, intensive and differed in length from the neighbouring fragments were analysed. The presence or absence of products were treated as individual feature and recorded as 1 or 0. Similarity index was estimated using the Dice coefficient of similarity (DICE 1945). Dice coefficient = $(x,y) = 2P(x, y)/(P(x) + P(y)$; were $P(x)$ and $P(y)$ is the probability of events x and y setting together. The dendrogram was constructed by unweighted pair group method with arithmetic averages (UPGMA; Diversity database; BioRad, USA).

SSR analysis. The number of individual (N) and number of effective alleles (Ne) of SSR loci in each population as well as for all populations simultaneously were calculated. Variability for each locus was measured using the polymorphism information content (PIC) (ANDERSON et al. 1993).

$$PIC = 1 - \sum_{i}^{n} p_i^2$$

where:
p_i is the frequency of the ith allele.

The number of different alleles across nine SSR loci in populations (Na), observed heterozygosity (Ho), expected heterozygosity (He), were estimated. The data of microsatellite allele frequency were applied to calculate the unbiased genetic distance and genetic identity estimates by employing Nei genetic distance (NEI et al. 1983). Pairwise genetic distance between individuals as well as between populations were estimated from proportion of shared alleles approach (O'BRIEN 1991). Basing on calculated coefficients individuals as well as populations were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA). The relationship among popula-

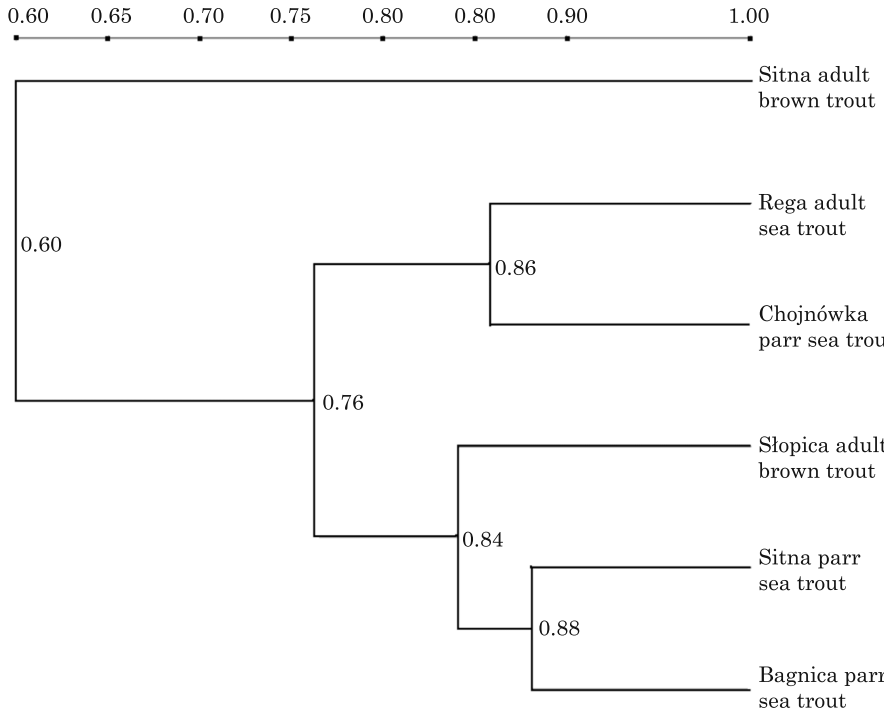
tions were presented in the form of a dendrogram. The resulting SSRs markers were used as input to a bootstrap procedure. SSRs were block-bootstrapped, with each SSR-locus representing one block. UPGMA-clusters were calculated for each of the 1000 bootstrap re-samples and a consensus tree was drawn.

Results

RAPD results. The number of bands (586) included the polymorphic loci. The number of fragments amplified by a single primer varied from 6 in C02 to 57 in C13, with the mean of 83.7 polymorphic products per primer. The percentage of polymorphic products ranged from 23.89% in the trout parr population from the Chojnówka to 45.39% in the trout parr from the Sitna stream (Table 2). As a result of reactions performed with the DNA of the brown trout and the sea trout, 100% polymorphic products were obtained. This result indicates a very high genetic variability of the trout populations studied. Based on the RAPD results, a similarity matrix was constructed and a tree of genetic similarity was drawn (Figure 2). The results of the RAPD analysis permitted the evaluation of genetic similarity between the investigated populations, with the results varying from 47.3% between the brown trout populations from the Słopica and Sitna streams to 88.1% between the sea trout parr from the Bagnica and those from the Sitna (Table 3). The UPGMA dendrogram reveals a division of the investigated populations into two similarity groups: the first group contains only the population of the brown trout from the Sitna, while the second group contains all the other populations. The second group is

Table 2
Characteristics of the RAPD markers polymorphism in parr sea trout, adult brown trout and adult sea trout from Bagnica, Słopica, Chojnówka, Rega i Sitna

Primer	Number of fragments		Number of polymorphic fragments					
	total	polymorphic	Bagnica parr sea trout	Słopica adult brown trout	Chojnówka parr sea trout	Rega adult sea trout	Sitna adult brown trout	Sitna parr sea trout
C02	81	81	22	29	16	32	6	27
C04	60	60	29	24	25	38	17	20
C06	99	99	45	54	21	29	15	57
C11	70	70	44	38	7	15	25	41
C13	91	91	44	36	29	57	20	38
Total	401	401	184	181	98	171	83	183
Polymorphic bands [%]			38.90	40.27	23.89	43.34	27.47	45.39



Numbers at the nodes indicate the percentage of similarity

Fig. 2. Dendrogram (UPGMA) showing the genetic relationships between analysed populations based on RAPD polymorphism, constructed based on the Dice coefficient of similarity

Table 3

Genetic similarity matrix (UPGMA) of the investigated population based on the PCR-RAPD analysis

River and individuals	Bagnica parr sea trout	Słopica adult brown trout	Chojnówka parr sea trout	Rega adult sea trout	Sitna adult brown trout
Słopica adult brown trout	84.9	–	–	–	–
Chojnówka Parr sea trout	77.0	68.0	–	–	–
Rega adult sea trout	79.8	69.6	85.8	–	–
Sitna adult brown trout	54.4	47.3	71.6	69.2	–
Sitna parr sea trout	88.1	83.2	79.1	83.7	57.3

subdivided into two subgroups: one consisting of the population of the sea trout from the Rega and the trout parr from the Chojnówka, while the other consisting of the brown trout populations from the Słopica and the trout parr from the Bagnica and Sitna streams.

SSR results. In the analysed populations, 25 alleles in 9 microsatellite loci were described. The number of alleles per locus ranged from 1 to 5, with

a mean of 2.8 alleles over all loci (Table 4). The polymorphic information content (PIC) of the SSR loci ranged from 0.439 to 0.921, with a mean of 0.815 over the nine loci. The observed heterozygosity per locus ranged from 0.02 to 0.78, with a mean of 0.37 over the nine loci (Table 4).

Table 4

Locus, allels number, PIC and expected and observed heterozygosity

Locus	No. alleles	PIC	Expected heterozygosity	Observed heterozygosity
Strutta 12	5	0.656	0.66	0.78
Str85INRA	4	0.792	0.59	0.57
Str60INRA	3	0.898	0.42	0.53
BS131	2	0.881	0.31	0.22
Strutta 58	4	0.873	0.47	0.47
Ssa197	1	0.921	0.09	0.12
Str43INRA	1	0.439	0.07	0.02
Ssa 85	3	0.970	0.33	0.36
SsoSL417	2	0.901	0.23	0.25
Mean	2.8	0.815	0.35	0.37

The percentage of polymorphic loci in the analyzed populations ranged from 44.44% to 100.00% in the Rega (Table 5).

The tree (Figure 3) revealed a division of the investigated populations into two groups: one from the Rega river and the other from the remaining watercourses. The Sitna adult brown trout and parr trout made one subgroup.

Table 5

Percentage of Polymorphic Loci

Population	% of P
Bagnica parr sea trou	44.44
Słopica adult brown trout	66.67
Chojnówka parr sea trout	55.56
Rega adult sea trout	100.00
Sitna adult brown trout	66.67
Sitna parr sea trout	66.67
Mean	66.67
SE	7.59

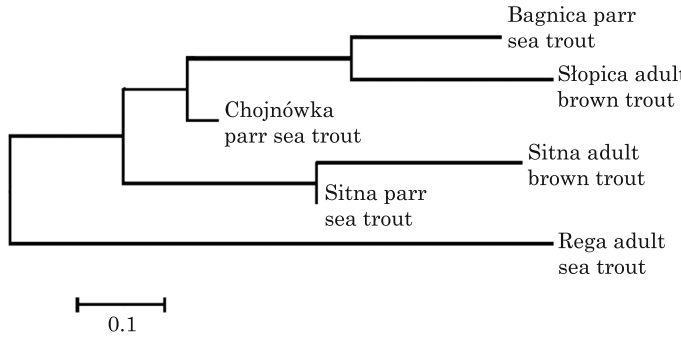


Fig. 3. Dendrogram (UPGMA) showing the genetic relationships between analysed populations based on SSR polymorphism; the 1000 bootstrap re-samples

Discussion

An important problem in the studies of fish populations is the recognition of the range of occurrence, as well as the intra- and interpopulation variability which can be affected by environmental conditions and/ or selection pressure. The relevant data are obtained with the help of different marker systems. We attempted to compare the genetic variability of the populations of *Salmo trutta* m. *fario* and *Salmo trutta* m. *trutta* from three watercourses in the Drawa river catchment area: Sitna, Słopica and Bagnica, and from the Rega and Chojnówka rivers, using a method based on the analysis of microsatellite sequences and RAPD-PCR. The genetic variability of the brown trout was evaluated earlier on the basis of morphological and, subsequently, isoenzyme studies (GIUFFRA et al. 1996, OSINOV 1984, PRESA and GUYOMARD 1996), as well as mtDNA analysis (FRITZNER et al. 2001, HANSEN et al. 2000, LAIKRE et al. 2002, LARGIADER and SCHOLL 1996). Recently, the genetic structure of populations (OLSEN et al. 1998, TESSIER and BERNATCHEZ 1999) or the size of populations and changes in allele frequencies (BOWCOCK et al. 1997) have been increasingly often studied by microsatellite sequences (FOO et al. 1995, FRITZNER 2001, RUZZANTE et al. 2001). Moreover, the RAPD-PCR analysis has been often used for the determination of genetic variability and the degree of similarity of fish species (HATANAKA and GALETTI 2003), as well as for the differentiation of geographically isolated fish populations and for the verification of locally fitted populations of species potentially resulting from genetic selection under environmental pressure or genetic drift (FUCHS et al. 1998, SKUZA et al. 2009).

The RAPD analysis revealed different grouping and genetic similarity relations between the investigated *Salmo trutta* populations. The most genetically distant group was that of the brown trout from the Sitna, while all the other fish populations formed another, second group. In this second group, the greatest similarity, varying from 83.2 to 88.1%, was noted between the trout parr from the Sitna and Bagnica, and the brown trout from the Słopica, which can be explained partially by the location of these watercourses in the same catchment area of the Drawa river or by a common origin. Such a high genetic similarity between fish from different watercourses has been reported by GARCI-MARIN and PLA (1996) and by DUNNER et al. (2000) for the indigenous Spanish population of the brown trout. These groups also used the RAPD-PCR method to determine the genetic variability related with the geographic localisation of *Salmo trutta* L. in three rivers in Spain (DUNNER et al. 2000). High similarity was also observed between the sea trout from the Rega and the trout parr from the Chojnówka (85.8%), which may very probably be due to a common origin of these two populations. The lowest similarity of only 47.3% was noted between the brown trout from the Słopica and the brown trout from the Sitna. This fact can be surprising, taking into account the small distance of about 10 km between these two watercourses. However, as mentioned above, the watercourse Słopica could be occupied by a local population. Besides, the Słopica joins the Drawa river at the section, which satisfies the needs of the salmonids that can freely migrate, while the Sitna joins Lake Adamowo that is a limnetic reservoir.

The analysis of the results obtained with the SSR primers indicated a high genetic popumorphism between the Rega population and the rest of the investigated populations (Figure 3). The values of the level of polymorphism (Table 2) and the heterozygosity (Table 4) were quite high. The same results were been reported by PRESA et al. (1994). In comparison with the results by ROLLINS et al. (2009), the mean number of alleles per locus is quite low.

The unaccounted for high genetic variability established in our study can be also related with the influence of ecological, evolutionary or historical factors. For instance, the polymorphism of *Barbus neumayeri* was explained by oxygen selective pressure (CZERNIAWSKI et al. 2010), high genetic differences observed between two populations of *Oncorhynchus nerka*, related with dwelling in different environmental conditions (HENDRY et al. 2000).

The study can be helpful in the concept of conservation of these species. Streams flowing into the Drava River before the construction of the dam in Głusk were a natural habitat of the trout. The streams, as some of the very few watercourses, still meet the living conditions required by salmonids. Among the existing resources are the fish populations in the buffer zone of DPN, not exploited to date because of their are small size and protected status. We could

not establish from which of the parents came the fry. Oral information received from fishermen confirmed the possibility of restocking individuals within several decades. To conclude, the presented results imply that the investigated fish species coming from the majority of watercourses show some genetic similarity with a high probability, therefore, it cannot be excluded that they could originate from the stocking material from the Rega river.

Translated by KAROL BOCIEK

Accepted for print 1.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 177–188, Y. 2014

TOURISM AND LEISURE AT LOWER SILESIAN NATURA 2000 SITES ON THE BASIS OF STANDARD DATA FORMS

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K e y w o r d s: tourism, leisure, Natura 2000 sites, Lower Silesia province, Standard Data Forms.

A b s t r a c t

Natura 2000 sites can be very attractive for leisure and tourism activities. However, these activities can have a negative impact on protected habitats and species. The main aim of this study was to analyze what forms of tourism and recreation have been developed at Lower Silesian Natura 2000 sites, and what are kinds of their impact and what is their intensity. The main research materials are Standard Data Forms. Among the 13 main types of tourism and leisure activities, found in analyzed sites, hiking, cycling, leisure fishing, hunting and their related infrastructure are the most frequently recorded.

The results show that low intensities are most often described. The frequency of occurrence of neutral or negative environmental impacts of these activities is the same. However, no examples of positive impact have been recorded.

TURYSTYKA I REKREACJA NA DOLNOŚLĄSKICH OBSZARACH NATURA 2000 W ŚWIETLE STANDARDOWYCH FORMULARZY DANYCH

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Abstrakt

Obszary Natura 2000 cechują cenne walory do rozwoju turystyki i rekreacji. Jednakże te formy aktywności mogą wpływać niekorzystnie na chronione siedliska oraz chronione gatunki roślin i zwierząt. Celem pracy było zbadanie, na przykładzie województwa dolnośląskiego, form turystyki i rekreacji, rodzaju ich oddziaływań na środowisko oraz intensywności tych oddziaływań. Materiałem źródłowym były standardowe formularze danych. Wśród 13 głównych form działalności turystyczno-rekreacyjnej, zidentyfikowanych na badanym obszarze, najczęściej wskazywano na oddziaływania związane z turystyką pieszą i rowerową (oraz infrastrukturą do ich rozwoju niezbędną), a także z wędkarstwem i myślistwem.

Wykazano, że dominują oddziaływania o niskiej intensywności. Rodzaje wszystkich stwierdzonych oddziaływań na przyrodę (neutralnych i negatywnych) rozkładają się po równo. Brak jest z kolei oddziaływań pozytywnych.

Introduction

The European Ecological Network Natura 2000 is a coherent strategy of nature conservation in all European Union Member States. The object of the program is to ensure the long-term survival of Europe's most valuable and threatened species and habitats, while taking into consideration economic, social, cultural, and regional requirements at the national level (EVANS 2012). This aim is in accordance with the European Council's goal of halting biodiversity decline (Commission of the European Communities 2006, European Commission 2011a) and fulfils a Community obligation outlined under the UN Convention on Biological Diversity (United Nations 1992).

The network is based on two EU directives: the Birds one and the Habitats one. The first is Council Directive 79/409/EEC of 2 April 1979 on the conservation of wild birds (commonly known as the Birds Directive), which was superseded with a new consolidated version in 2009 (European Communities 1979, 2009). This Directive refers to specific bird habitats as Special Protection Areas (SPAs). The Habitats Directive, the official name of which is Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora (European Communities 1992), initially lead to establishing of a list of proposals. After evaluation and selection process on European level, each site is referred to as Site of Community Importance (SCIs), which is then eventually designated as Special Area of Conservation (SACs) by each EU Member State.

Currently, the Natura 2000 network comprises over 27 221 sites and covers 18.16% of the territory of all 28 EU Member States (European Commission 2014). The network seems to be the best integrated vision of nature conservation in European countries, which is a key issue in terms of effective management (NOLTE et al. 2010). It is also the largest network of protected areas in the world (SUNDSETH and CREED 2008).

The European Ecological Network Natura 2000 has been functioning in Poland since 2004, when the country accessed the European Union. The Natura 2000 program differs considerably from the traditional Polish protection system in that there are no *a priori* prohibitions and obligations of new activities or developments within designated sites (HABUDA 2013). According to Polish law, only those human activities can be forbidden that, which single or together with other ones, leave or could possibly leave a significant negative impact on protected subject-matters of Natura 2000 sites (Nature Conservation Act, Article 33, point 1).

The General Directorate for Environmental Protection (GDEP) and its representatives in each voivodeship (province), i.e. Regional Directorates for Environmental Protection (RDEPs), are responsible for the implementation of the Natura 2000 strategy and management of these areas. However, the monitoring of flora and fauna species are tasks of Inspection for Environmental Protection (IEP). Fulfillment of these responsibilities is often seen to be in conflict with the interests of local communities and stakeholders in most EU countries (JULIEN et al. 2000, Commission of the European Communities 2004, PALONIEMI and TIKKA 2008, KEULARTZ 2009, GRODZINSKA-JURCZAK and CENT 2011). In some new EU Member States, such as Poland, the designation and implementation of the Natura 2000 program is still a source of social conflicts (PIETRZYK-KASZYNSKA et al. 2012, WOŁOSZYN et al. 2012). As in other Central and Eastern European countries, the Natura 2000 system in Poland is often perceived as a threat to local and regional socio-economic development (GRODZINSKA-JURCZAK et al. 2012).

Currently, the Natura 2000 network comprises over 983 sites and occupies 19.6% of the territory of Poland (European Commission 2014).

Each Natura 2000 site has got its own documentation. The basic documents, obligatory for all sites, are the Natura 2000 Standard Data Form (SDF) and a digital map. Article 6.1 of the Habitat Directive defines the conservation measures that are required to be taken, among them establishing management plans (European Communities 1992). Although Natura 2000 site management plans are recommended, they are not obligatory in all EU Member States (EVANS 2012). In Poland, 10-year plans of protection tasks or, if it is necessary, 20-year protection plans have to be developed for both SPA and SAC areas. Due to the complicated procedures and high decision-making costs (WÄTZOLD et al. 2010), only 6.5% Polish Natura 2000 sites have had their management plans approved since 2004 (Platforma komunikacyjno-informacyjna 2014). Therefore, Standard Data Forms are often the only documents in which it is possible to find some information concerning impacts and activities influencing the conservation status of protected subject-matters.

130 types of human activities have been recognized which can influence Natura 2000 sites. 'Leisure and tourism' is one of the most frequently reported category (TSIAFOULI et al. 2013). Tourism, one of the largest and fastest-growing economic sectors in the world (UNWTO 2013), is perceived as a key driver of socio-economic progress, also at Natura 2000 areas (PRÖBSTL 2003, KAMIENIECKA and WÓJCIK 2010). However, unsustainable tourism is also associated with negative effects (PSTROCKA and RAK 2006).

The main goal of this study was to determine, on the basis of Standard Data Forms, human activities and impacts connected with tourism and leisure within Natura 2000 areas and their surroundings. In particular, the interest in this study has focused on:

1. What kind of human activities and impacts are recorded?
2. What is their frequency of occurrence?
3. What are kinds of their impacts and intensity?
4. How can areas most exposed to impacts be best defined?

Materials and Methods

The main research materials were Natura 2000 Standard Data Forms (SDF) completed for Lower Silesia province. As they are updated frequently, SDFs are one of the key instruments in the effective management of nature conservation (Nolte et al. 2010). SDFs provide information on the conservation status of the protected habitats and species at SCI/SAC and SPA sites. Together with information on the species and habitats SDFs include general information on the site (location, date of designation and updating, site description, relation with Corine biotope sites etc.) and on impacts and activities in and around the site (European Commission 1997). In 2011 a new revised version of SDF was approved by the Habitats Committee (European Commission 2011b). The first new SDFs for Polish Natura 2000 sites were accepted in May 2013 (Generalna Dyrekcja Ochrony Środowiska 2014). There were only 14 new SDFs (out of 100) for Lower Silesia province when we started this analysis (December 2013). For this reason and due to important changes between the new and old versions of SDFs, only old ones were analysed. The main source of Standard Data Forms has been the official web site of the General Directorate for Environmental Protection (Generalna Dyrekcja Ochrony Środowiska 2014).

Human activities and impacts are classified into 130 types and grouped in eight categories (European Commission 2000). One of these is 'Leisure and tourism', where 20 types of impacts and activities influencing the conservation status of a site have been distinguished. Nevertheless, there are some other

tourism and leisure activities and impacts which have been included under different headings. Paths, tracks, cycling tracks (code 501), leisure fishing (code 220) and hunting (code 230) have been added to the above-mentioned group. Fishing and hunting are primarily recreational activities in the majority of EU-protected areas, in contrast to protected areas on other continents, such as Asia or Africa, where these activities are engaged in mainly for subsistence or livelihood purposes (TSIAFOULI et al. 2013). Activities and impacts which are not directly connected with tourism and leisure, e.g. autoroutes, railway lines, and vandalism, have not been analyzed in detail.

The main research method was analysis of information given in the SDFs, in particular:

– impacts observed in Natura 2000 sites and their intensities related to tourism and leisure activities that may have an influence, either positive, neutral or negative;

– the impacts and activities in the surroundings of the Natura 2000 site. The surroundings comprise the area where external impacts and activities may affect the integrity of the site (European Commission 2000);

– features of the sites, where a negative influence is medium or high.

The study area was Lower Silesia province, located in the south-western part of Poland. The most characteristic features of the region include well developed and varied industries and agriculture, as well as related urbanization. These characteristics have been influencing the nature of the area since the eighteenth century, when the Industrial Revolution started. Concurrently Lower Silesia is a region of very different types of mountainous and lowland environment, which together with its cultural assets make the region very attractive for mass tourism. The tradition of tourism development dates back to the nineteenth century, and even the Middle Ages for spas. Simultaneously, according to GDEP data, the province ranks 3rd in Poland in terms of numbers of Natura 2000 sites.

There are 100 Natura 2000 sites in Lower Silesia province. Ten Special Protection Areas (SPAs) cover 18.1% of the province's territory. There are ninety areas linked to the Habitats Directive, which occupy 19.1% of Lower Silesia (natura2000.gdos.gov.pl). Due to the fact that SPAs comprise only 10% of all numbers of Natura 2000 sites, they were treated together.

Results and Discussion

In Lower Silesia province 92 categories of impacts and activities have been recognized, out of the 130 types formulated in the explanatory notes for SDF. Natural processes comprise only 15% of the analyzed impacts. The remaining

78 types are connected with human activities. These have been studied further.

There are 79 Natura 2000 sites (out of 100) in Lower Silesia where some general impacts or human activities have been determined. 'Agriculture and forestry' and 'Leisure and tourism' are the most frequently reported categories of human activities at the analyzed sites (Figure 1).

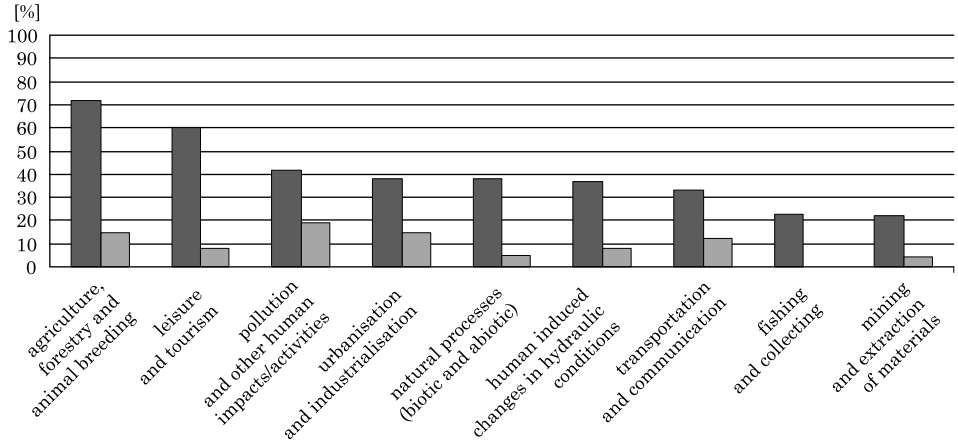

Fig. 1. Distribution of categories of impacts and human activities at Natura 2000 sites (dark colour bars) and in their surroundings (bright colour bars)

In the case of activities in the surroundings of Natura 2000 sites 'Pollution and other human impacts/activities' is the most often recorded category. These impacts occur in 19% of all Natura 2000 sites. Activities related to 'Leisure and tourism' concern only 8 sites (Figure 1).

Subdividing general categories, we can distinguish 22 individual impacts and activities which have occurred at 10% or more Lower Silesian sites (Table 1). None of them have exceeded this frequency of occurrence in the surroundings of Natura 2000 sites. The most often recorded impacts there were connected with routes and autoroutes (code 502) and water pollution (701), which concern only 9 sites each.

Leisure and tourism impacts have been found at 60 Natura 2000 sites (133 records) and in 8 their surroundings (15 records). Some of these concern the influence of infrastructure (especially paths, tracks, cycling tracks – code 501) while others are connected with human activities: hunting (230), leisure fishing (220), walking and horse-riding (622). These are the most often recorded (Figure 2).

Table 1

Impacts and human activities at Natura 2000 sites

Code	Impacts and human activities	Distribution of impacts and human activities at Natura 2000 sites [%]
160	general forestry management	44.0
501	paths, tracks, cycling tracks	30.0
102	mowing/cutting	29.0
166	removal of dead and dying trees	26.0
502	routes, autoroutes	26.0
954	invasion by a species	23.0
164	forestry clearance	22.0
230	hunting	22.0
701	water pollution	22.0
220	leisure fishing	21.0
141	abandonment of pastoral systems	19.0
403	dispersed habitation	19.0
622	walking, horse-riding and non-motorised vehicles	18.0
100	cultivation	17.0
620	outdoor sports and leisure activities	17.0
101	modification of cultivation practices	16.0
161	planting	16.0
301	quarries	16.0
421	disposal of household waste	16.0
140	grazing	13.0
702	air pollution	13.0
180	burning	12.0
952	eutrophication	11.0
200	fish and shellfish aquaculture	10.0

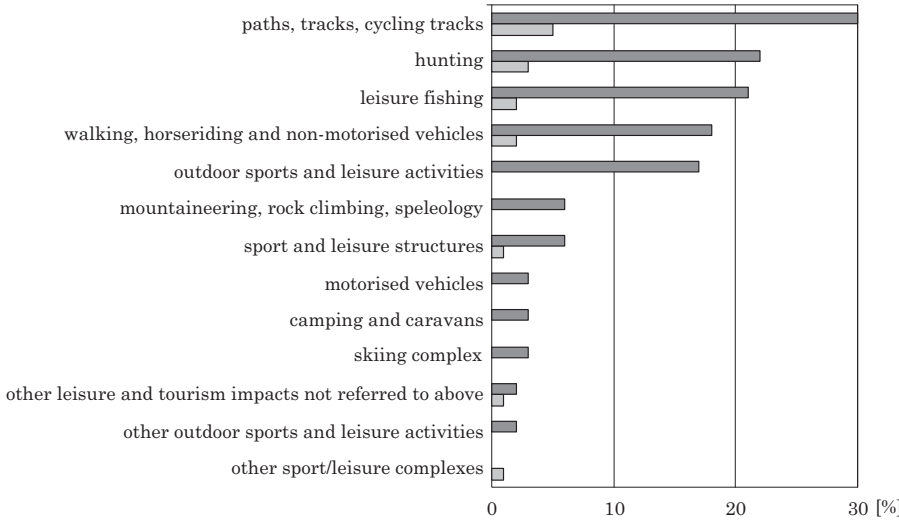

Fig. 2. Distribution of 'Leisure and tourism' impacts and activities at Natura 2000 sites (dark colour bars) and in their surroundings (bright colour bars)

Furthermore these leisure and tourism activities and infrastructure can have different environmental impacts: negative, positive or neutral. These influences can also be described in terms of their level of intensity: high, medium or low. According to the authors of Lower Silesian SDFs, there are no positive impacts either inside or outside any sites. Half of the authors cite negative impacts on habitats and species. The influence of the same number of activities is regarded as neutral by them (Table 2). Most leisure and tourism impacts (63.2%) are kept low. Only 6% of negative impacts are at the highest level of intensity.

Table 2
Influence and intensity of 'Leisure and tourism' activities at Natura 2000 sites in Lower Silesia province

Specification		Intensity of the influence				
		high (*A*)	medium (*B*)	low (*C*)	total	% of all records
Influence	positive (+)	0	0	0	0	0.0
	neutral (0)	2	15	49	66	49.6
	negative (–)	8	24	35	67	50.4
	total	10	39	84	–	
	% of all records	7.5	29.3	63.2		

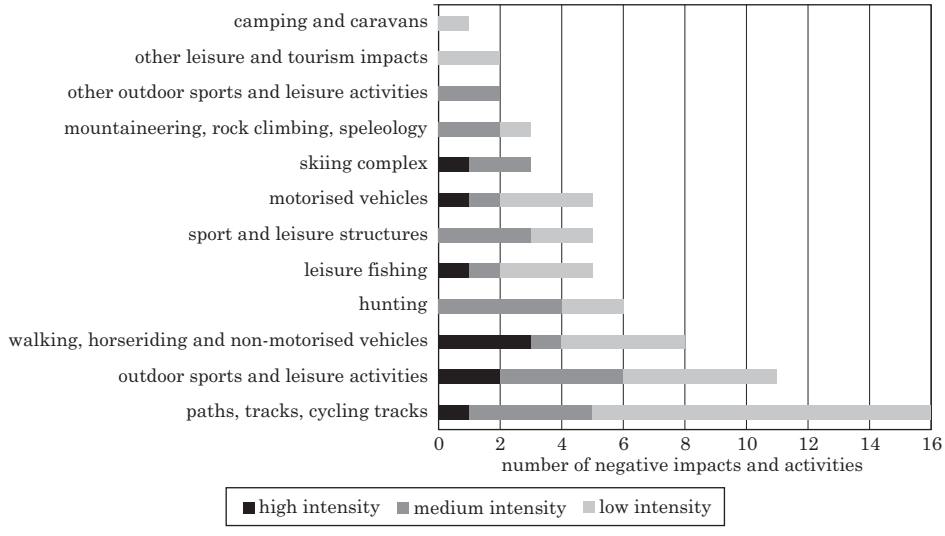

Fig. 3. 'Leisure and tourism' negative impacts and activities at Natura 2000 sites

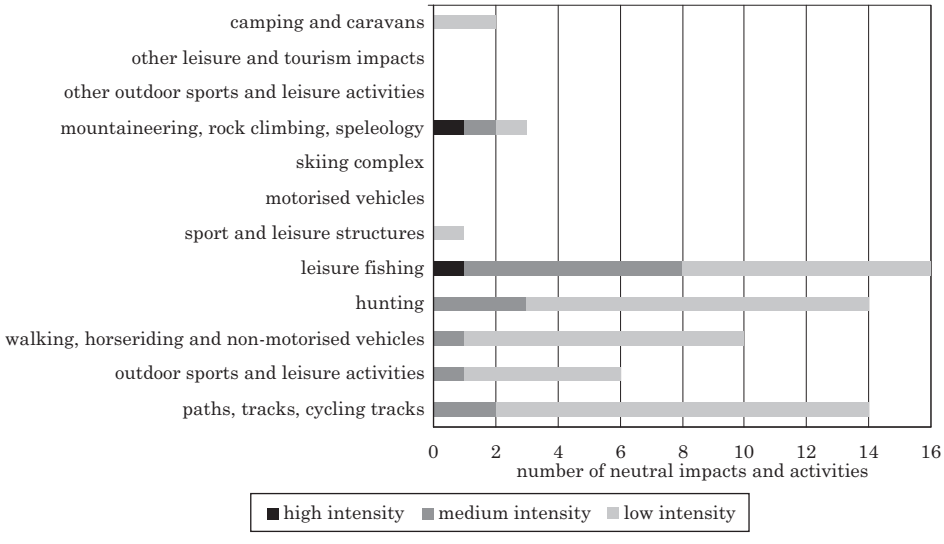

Fig. 4. 'Leisure and tourism' neutral impacts and activities at Natura 2000 sites

Figure 3 and Figure 4 show that the same structure elements (e.g. trails) or the same activities (leisure fishing, hunting, walking etc.) can have different impacts (negative or neutral) regardless of their intensity. The reason is that the evaluations of the influence are carried out on particular habitats and species (different subject-matters of Natura 2000 sites), in contrast to general assessments of the environmental impact of tourism and leisure. However,

there are some impacts which are described only as negative, such as skiing complexes and motorized vehicles.

Studying the data in detail, it is noticeable that medium and high intensive negative influences are mostly observed at medium-sized (average area: 19519.4 hectares) mountainous Natura 2000 sites with a wide variety of habitats (both wildlife and semi-natural) and species (mean number: 19.1 per site), e.g. Karkonosze (PLH020006, PLB020007), Góry Stołowe (PLB020006), Góry Złote (PLH020096), Góry Orlickie (PLH020060), Masyw Ślęży (PLH020040). These areas can also be characterized as being very attractive to tourists.

The sites where the influence is mainly low and neutral are usually small-sized (average area: 3986.7 hectares) with a fewer number of species (mean number: 10.2 per site) related to a narrow type of environment and with some leisure attractions, mostly for local residents, e.g. Czarne Urwisko koło Lutyni (PLH020033), Dąbrowy Kliczkowskie (PLH020090), Góra Wapienna (PLH020095), Kamionki (PLH020005).

The results of the most often reported categories of human activities correspond with the results of similar research carried out in 20 EU Member States (TSIAFOULI et al. 2013). They show also the significant role of tourism and leisure activities at Natura 2000 sites.

The analysis showed that the main types of leisure and tourism impacts at Natura 2000 sites are exactly the same for Lower Silesia province and for the Polish part of the Carpathian region. In spite of the fact that SDFs were analyzed for two different areas of Poland, the low intensity and division of types of environmental impact (neutral and negative) are very similar (WITKOWSKI et al. 2012).

Conclusions

The Lower Silesian Natura 2000 sites are frequently attractive for nature conservation as well as for leisure and tourism activities. There are 13 main types of tourism and leisure impacts. The most frequently recorded activities were hiking, cycling, leisure fishing and hunting. However, these activities and the infrastructure necessary to develop them influence the environment inside and outside the sites. Nevertheless, in view of research results of standard data forms their type and intensity are not often harmful. A high intensity of negative environmental influence is rare. Overall, we can conclude that these two important functions of Natura 2000 sites, i.e. nature conservation and tourism/leisure, can be fulfilled simultaneously in the Lower Silesia province.

Translated by MAŁGORZATA PSTROCKA-RAK

Accepted for print 21.07.2014

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 29(2): 189–195, Y. 2014

SUBCLINICAL *ANGIOSTRONGYLUS VASORUM* INFECTION IN A TERRIER DOG KENNEL

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K e y w o r d s: *Angiostrongylus vasorum*, dogs, diagnosis, imidacloprid, moxidectin, Italy.

A b s t r a c t

Angiostrongylus vasorum is a parasitic nematode causing severe clinical signs in infected dogs. In the past few years *A. vasorum* has been repeatedly described in both traditional endemic foci and previously free regions. Nonetheless, the infection is often neglected or unnoticed by vet practitioners, due to gaps of information on *A. vasorum* epidemiology, and to drawbacks inherent to the clinical and parasitological diagnosis. Indeed, subclinical infections may occur and, when present, clinical signs are difficult to differentiate from those of other canine cardio-pulmonary diseases. Additionally, the *gold standard* test for the aetiological diagnosis of the infection, i.e. the Baermann's method, is not commonly performed by veterinarians.

The present study describes cases of subclinical *A. vasorum* infection in a Jack Russell Terrier dog kennel in Italy and the ability of a newly marketed rapid kit (IDEXX *Angio Detect™ Test*) for the field diagnosis of angiostrongylosis, pre- and post-treatment with a formulation licensed for the treatment of *A. vasorum*.

SUBKLINICZNE ZARAŻENIE *ANGIOSTRONGYLUS VASORUM* W HODOWLI PSÓW TERIERÓW

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S ł o w a k l u c z o w e: *Angiostrongylus vasorum*, psy, diagnostyka, Imidacloprid, Moxidectin, Włochy.

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Abstrakt

Angiostrongylus vasorum to pasożytniczy nicień powodujący ciężkie objawy kliniczne u zarażonych psów. Na przestrzeni ostatnich kilku lat wielokrotnie opisywano przypadki inwazji *A. vasorum* zarówno w endemicznych ogniskach, jak również w regionach wcześniej wolnych od tego pasożyta. Często jednak zdarza się, że możliwość inwazji zostaje pominięta lub przypadek zarażenia pozostaje niedostrzeżony przez lekarza weterynarii z powodu niedostatecznej wiedzy na temat epidemiologii *A. vasorum* oraz niedociągnięć czy błędów podczas badania klinicznego i parazytologicznego. Podkliniczna postać inwazji może jednak występować – w takiej sytuacji objawy kliniczne są trudne do odróżnienia od tych towarzyszących innym chorobom układu sercowo-naczyniowego i chorobom płuc u psów. Co więcej, standard diagnostyki etiologicznej inwazji, czyli metoda Baermanna, nie jest powszechnie wykorzystywana przez lekarzy weterynarii.

W pracy opisano przypadki podklinicznej inwazji *A. vasorum* w hodowli Jack Russel terierów we Włoszech oraz podjęto zagadnienie możliwości zastosowania nowo wprowadzonego na rynek testu (IDEXX *Angio Detect™ Test*) w diagnostyce terenowej angiostrongylozy przed leczeniem preparatem dopuszczonym do stosowania w terapii inwazji *A. Vasorum* i po takiej kuracji.

Introduction

Canine angiostrongylosis is an emerging parasitic disease of dogs and wild animals (e.g. foxes and wolves) caused by *Angiostrongylus vasorum* (Nematoda, Metastrongyloidea). The interest on this nematode is growing due to the severe clinical outcome of the infection in dogs, the increase of reports in both traditional endemic foci and previously free areas, and for the recent insights achieved on the biology, treatment and the diagnosis of the infection (FERDUSHY and HASAN 2010, TRAVERSA et al. 2010, 2013, SCHNYDER et al. 2014). Adult stages reside in the heart and pulmonary arteries, where adult females produce eggs that embryonate and hatch within alveolar ducts and alveoli. First stage larvae (L1) penetrate into the alveoli and migrate to the pharynx, are swallowed and released into the environment *via* the feces. The life cycle of *A. vasorum* is indirect, involving slugs and snails as intermediate hosts, in which L1 develop to the third infectious stage (L3). Dogs become infected by ingesting mollusks harboring L3 (ANDERSON 2000, FERDUSHY and HASAN 2010, MORGAN and SHAW 2010). *Angiostrongylus vasorum* has been considered for a long time to be present only in well-isolated endemic foci (i.e. areas of France, UK and Denmark). However, in the last decade, the nematode has been recorded in dogs in previously free areas of northern and central Europe, e.g. Sweden, Switzerland, Germany, of the Mediterranean Basin, e.g. Greece and Italy, and of Eastern Countries, e.g. Slovakia, Poland, Hungary (MAJOROS et al. 2010, TRAVERSA et al. 2010, 2013, DI CESARE et al. 2011, HURNÍKOVÁ et al. 2013, GUARDONE et al. 2013, SCHNYDER et al. 2013).

Canine angiostrongylosis may be asymptomatic/sub-clinical or characterized by iper-acute, acute or chronic signs, that may be life-threatening when a specific therapy is not administered (TRAVERSA and GUGLIELMINI 2008,

FERDUSHY and HASAN 2010, MORGAN et al. 2010, TRAVERSA et al. 2010). Cardiac, neurologic, gastrointestinal and hematological signs may be present in infected animals, being coughing, dyspnea, and some non-specific signs, such as anorexia and weight-loss, lethargy, depression the most common. Cardiovascular signs (e.g. heart murmur, ascite, syncope) of congestive heart failure (*cor pulmonale*) may be observed. Bleeding disorders and coagulopathies may cause petechial or ecchymotic haemorrhages in the conjunctiva, episclera, gingiva and subcutis, as well as epistaxis, haemoptysis, post-surgical haematomas, gastrointestinal bleeding, haematuria and anaemia. Others common signs of angiostrongylosis include neurological (e.g vestibular signs, convulsion and paralysis), ocular signs (e.g. uveitis) due to larvae or egg embolism or haemorrhages. Infected dogs may also show vomiting, diarrhoea and anorexia (GOULD et al. 1999, CHAPMAN et al. 2004, OLIVEIRA-JÚNIOR et al. 2004, TRAVERSA and GUGLIELMINI 2008, TRAVERSA et al. 2008, 2010, 2013, KOCH et al. 2009). A recent multi-centric survey performed in dogs with clinical pictures compatible with angiostrongylosis confirmed that non-specific gastrointestinal, respiratory, hematological and neurological signs may occur and that coughing is the most prevalent (TRAVERSA et al. 2013). Given the lack of specificity of signs, the clinical diagnosis of the infection is impossible. The detection of L1 in faeces of infected animals with the Baermann's method is the most reliable approach to achieve the aetiological diagnosis of angiostrongylosis (TRAVERSA and GUGLIELMINI 2008). This technique is relatively easy to perform and cheap, although it is time-consuming (i.e. 24–36 h) and requires well-trained microscopists. In fact, L1 should be recognized based on their length (i.e. 310–400 μm) and tail, having a typical sinus wave curve with a dorsal spine. These larvae need to be discriminated from those of other free-living or parasitic nematodes (i.e. *Crenosoma vulpis*, *Oslerus osleri* or *Filaroides* spp.) which can be present in canine faeces (TRAVERSA et al. 2010).

In additional, the Baermann's method has major disadvantages like the inability to diagnose infections during the pre-patent period and when larvae are not being shed, even in presence of severe clinical signs (CONBOY 2009, TRAVERSA et al. 2010). Innovative studies have been recently performed to overcome the constraints of copromicroscopic approaches (AL-SABI et al. 2010, SCHNYDER et al. 2011, 2013, 2014, SCHUCAN et al. 2012). After some of these studies, a newly marketed rapid kit (IDEXX *Angio Detect™ Test*) has been recently developed for the serological diagnosis of angiostrongylosis. The present study described cases of subclinical infections by *A. vasorum* in a Jack Russell Terrier dog kennel in Italy and the efficiency of this new kit in the field diagnosis of angiostrongylosis. The diagnostic performance of both the Baermann's test and the rapid kit has been evaluated before and after treatment with a parasiticide spot-on formulation licensed for the treatment of *A. vasorum*.

Materials and Methods

Study design and animals

The study was carried out in a private Jack Russell Terrier kennel located in Gallicano Municipality (Tuscany region, central Italy), selected for a previous history of angiostrongylosis. At Day -15 all the fifteen dogs living in the kennel were clinically examined and then faeces and blood were collected to be subjected, respectively, to the Baermann's method and to the detection of the circulating antigen of *A. vasorum* with the *Angio Detect™ Test*. Dogs were considered infected when positive at the Baermann's and/or the *Angio Detect™ Test*. At Day 0 positive dogs were clinically examined and treated with a spot-on formulation containing 10% imidacloprid and 2.5% moxidectin (Advocate®, Bayer). Two and four weeks after treatment (Days 14 and 28) these dogs were examined for clinical signs and samples were collected and examined as above with the Baermann's method and the *Angio Detect™ Test*.

Baermann methods and *Angio Detect™ Test*

The Baermann's test was performed as on the follow: 3–5 grams of each stool sample was put in the center of double layers of cheesecloth sheet. A pouch containing the fecal material was formed by holding the four corners of the cheesecloth sheet together and molding the cloth around the fecal material using a closing string. The pouch was placed in a funnel filled with water and kept at room temperature. After 24 hours, 15 ml of fecal fluid was drawn off the bottom funnel into a tube and centrifuged at 2000 rpm for 5 minutes. The sediment was transferred onto a slide and microscopically examined using a light microscopy at 10X, 40X and 100X.

Angiostrongylus vasorum L1s retrieved at the copromicroscopic examination were identified according to morphological and morphometrical keys features (TRAVERSA et al. 2010). The *Angio Detect™ Test* was performed and interpreted following manufacturer's instructions using the plasma collected from EDTA-blood samples.

Results and Discussion

At the clinical examinations performed before (Day-15) and after (Day 14 and 28) treatment no dogs showed clinical signs suggestive of angiostrongylosis.

The Baermann's test performed at Day-15 revealed the presence of *A. vasorum* L1 (Figure 1) in the faeces of 3 dogs. Two of them resulted positive also at the *Angio Detect™ Test* (Table 1). All examinations performed after treatment (Day 14 and Day 28) with the Baermann's methods and the rapid kit were negative (Table 1). The negative result of the kit in one infected dog at the pre-treatment evaluation is not indeed surprising. The possible explanations of this result could be the low level of circulating antigen due

Fig. 1. First stage larvae of *Angiostrongylus vasorum* (200x magnification)

Table 1

Results of investigations of fifteen dogs living in a dog kennel

Dog ID	D – 15		D – 14		D – 28	
	B	ADT	B	ADT	B	ADT
1	–	–	np	np	np	np
2	–	–	np	np	np	np
3	–	–	np	np	np	np
4	+	–	–	–	–	–
5	–	–	np	np	np	np
6	–	–	np	np	np	np
7	–	–	np	np	np	np
8	–	–	np	np	np	np
9	–	–	np	np	np	np
10	–	–	np	np	np	np
11	–	–	np	np	np	np
12	–	–	np	np	np	np
13	+	+	–	–	–	–
14	+	+	–	–	–	–
15	–	–	np	np	np	np

Explanation: Results of Baermann's method (B) and *Angio Detect Test IDEXX* (ADT) tests at days (D) – 15 pre-treatment with moxidectin and +14 and + 28 post-treatment with moxidectin. + positivity; – negativity; np: not performed.

an early stage of the infection (as supported by the lack of clinical signs) and/or the formation of antigen-antibody complexes, which may inhibit the detection of *A. vasorum* antigens, as described also for *Dirofilaria immitis* (SCHNYDER et al. 2014). Overall, it has been recently shown that this kit has a specificity of ~100% and a sensitivity of ~85%, which may lead to negative results in dogs positive at the BAERMANN'S test (SCHNYDER et al. 2014). Importantly, further studies on a large scale are necessary to evaluate in field conditions the concordance between the results of the Baermann's test and the rapid kit, in both clinically and subclinically infected dogs.

The results of the present study confirm the possible occurrence of subclinically infected dogs with no apparent signs compatible with the infection. A diagnosis of angiostrongylosis should be always considered in the presence of compatible clinical signs (TRAVERSA et al. 2013). However, dogs living in endemic areas, especially young animals and those that usually eat mollusks, should be routinely screened for *A. vasorum* even in absence of clinical signs. In fact, young dogs are more susceptible to the infection for their age-related level of immunity (TRAVERSA and GUGLIELMINI 2008, FERDUSHY and HASAN 2010). The *Angio Detec™ Test* is particularly suitable for this purpose, as it can be directly used in veterinary practices and it represents a valid tool for a quick diagnosis for its high values of sensitivity and specificity (SCHNYDER et al. 2014).

Furthermore, the *Angio Detect™ Test* is a powerful tool in dogs with clinical signs and needing a prompt anthelmintic treatment. In fact, this test may provide results in fifteen minutes, while the Baermann's test requires at least 24 hours. On the other hand, dogs presenting clinical signs compatible with angiostrongylosis but negative at the *Angio Detect™ Test*, should be examined with the Baermann's method before excluding the infection.

A reliable diagnosis of dog angiostrongylosis is of great importance under a practical standpoint. In fact, despite the severe pathogenic impact of *A. vasorum*, the available parasiticide options are straightforward and effective (TRAVERSA et al. 2010). Hence, it is noteworthy that the present study confirmed the high efficacy of moxidectin contained in Advocate® for the therapy of canine angiostrongylosis (WILLESEN et al. 2007).

Accepted for print 28.07.2014

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