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THE YELLOW-BILLED CHOUGH *PYRRHOCORAX GRACULUS* LINNAEUS, 1766 IN ARMENIA: UPDATE ON CONSERVATION STATUS

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Key words: Yellow-billed Chough, Alpine Chough, Pyrrhocorax graculus, Armenia, conservation status.

Abstract

The Yellow-billed Chough *Pyrrhocorax graculus* is one of the least-studied species in Armenia and was surveyed in 2003–2019. Results show that in Armenia the species has four major populations: in the north-eastern, central and south-eastern regions of the country, inhabiting sub-alpine zone at elevations range 1,800–3,000 meters above sea level. The area of occupancy of the species in Armenia is calculated as 186 km^2 and the population is comprised of at least 68 adult individuals. The primary threat comes from the disturbance of nesting pairs in the breeding season by extreme cave tourism. The species' conservation status should remain the same – Endangered, although the criteria should be modified into B2*a* and *D*. The current conservation measures are not sufficient and should include identification of other loose colonies and their inclusion into the protected area network, and minimization of disturbance to the species by extreme tourism. The further monitoring of this species remains essential.

Introduction

The Yellow-billed or Alpine Chough *Pyrrhocorax graculus* is a species that has patchy distribution in alpine regions of Europe, Caucasus, Middle East and Central Asia (CRAMP and Perrins 1994, Madge 2020). It is classified as Least Concern in the IUCN Global Red List having a stable population trend (BirdLife International 2015). Yellow-billed Choughs predominantly inhabit high-elevation mountain pastures with cliffs and crags above tree-line. In general, it is solitary nester, however forming of the

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loose colonies is also know, as well as colonial breeding (e.g. up to 20 pairs in Bulgaria), although the latest seems to be exceptional. The species constructs its bulky-structured nests on ledge near roof of cave or rock chimney or rock crevice, especially prefers larger caves with small entrance (MADGE 2020). In Armenia the single records in breeding season exist since 1959. Somewhat bigger numbers 50 birds in Southern and 35 birds in South-eastern Armenia were registered in July of 1995, and although their breeding remained unconfirmed, the species was listed as a breeding bird with low numbers (ADAMIAN and KLEM 1999).

Armenia is a country of South Caucasus, located between the Black and Caspian Seas, and thanks to large variation of elevation (from 375 to 4090 m above sea level), hosts wide variety of landscapes, including semi-desert, juniper woodland, deciduous forest, mountain steppe, and sub-alpine area (AGHABABYAN et al. 2015). The alpine areas with rocky outcrops seem to be the most suitable habitat for Yellow-billed or Alpine Choughs *Pyrrhocorax graculus* (ADAMIAN and KLEM 1999). Armenia is inhabited by the nominate subspecies *P. g. graculus* Linnaeus, 1766 (Adamyan and KLEM 1999, MADGE 2020), which is included in National Red Book as Endangered under category *D* (*The Red Book…* 2010) being known only from rocky areas and their environs in the subalpine and alpine zones of Zangezur mountain ridge in South-eastern Armenia, in low numbers. The current study is aimed at contributing new knowledge on this poorly-studied species in Armenia by providing additional data on its distribution and abundance, evaluating current and potential threats, and proposing a revision of its conservation status for the next edition of Red Book of Animals of Armenia, planned for writing in 2020–2022.

Material and Methods

At the beginning of study, few records of Yellow-billed Choughs from Armenia were known (ADAMIAN and KLEM 1999, *The Red Book…* 2010). In 2003 we have started systematic data collection during a general program on monitoring of breeding birds in Armenia. Monitoring was implemented using the line transects or points. We follow the guidelines of European Breeding Bird Atlas 2 (*A best practice guide…* 2008), and using the standard European Monitoring Grid 10 x 10 km have divided the territory of the republic into 374 squares.

Data on the species was obtained from two different sources: unstandardized observations (so called opportunistic data) and standardized counts (data, collected according to standard methodology). Both data may

be used to create species distribution maps and for estimation of the species population.

Unstandardized observations (opportunistic data) were provided by birdwatchers and contain minimum data requirements: precise identification of species, observation date, geographic coordinates, name of nearest locality (human settlement, mountain, historical site, etc.), breeding code, name of observer and his contacts. The observations have been commented, e.g. time, observation duration, number of people in the group, etc. Most of these data are stored at Biodiversity of Armenia subsite of the online platform Observation.org (https://observation.org), being retrieved upon necessity.

Standardized counts (counts conducted within certain time period) were led by both specialists and birdwatchers that have the required skills. Counts were implemented during fixed period of 1 or 2 hours, when an observer walked the route at a slow and constant pace. The best season for Alpine Chough count was considered the period between 1st of May and 30th of June, nevertheless, data collected later until July were used as well. Data collected during each sighting included the number of observed or heard individuals, observation date, geographical coordinates of a beginning and the end of the route, start and end times of the count, breeding code, name and contacts of observer/s. These collected data were entered into standardized protocol and later was inputted into National Database on Birds of Armenia owned by BirdLinks Armenia (former TSE) NGO. Since the breeding behavior of the species varies between solitary nester to loose colonial breeder, we estimated its population size by counting the maximum number of pairs or individuals in the flocks during the breeding season. Mapping was undertaken using ArcGIS 10.0 software and the area of occupancy calculated according to IUCN guidelines (IUCN Standards and Petitions Committee 2019). To estimate the threats, we conducted surveys of local farmers, hunters, rock-climbers, and cavers, as well as conducting interviews with the State Inspectorate for Nature Protection and Mineral Resources.

Results

Our investigations show that in Armenia the species forms four distinct population clusters in the north-eastern, central and south--eastern regions of the country (see the map on Figure 1). In all the regions the species was observed regularly during the breeding season, showing territorial behavior, i.e. staying at the same area throughout the breeding

season, demonstrating courtship behavior, having recently fledged young, and visiting the probable nesting places. Areas where breeding was most likely to occur are located in the subalpine zone at the elevation range 1,800–3,000 meters above sea level (Figure 2). In all those areas there are high cliffs with numerous small caves, niches, chimneys, and grottos; in some of the territories the potential nest sites could be large underground caves.

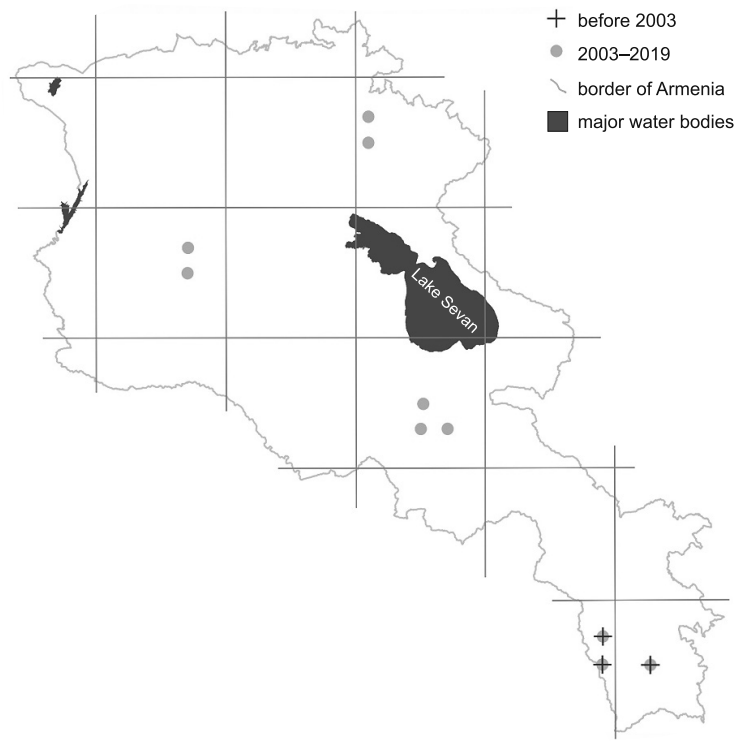

Fig. 1. Distribution of yellow-billed chough in Armenia

Fig 2. Typical habitat of Yellow-billed Chough in North-Eastern Armenia (photo by author)

The area of occupancy of the species in Armenia was calculated as 186 km^2.

According to our last count there are at least 68 adult individuals in the country. Unfortunately, the data is not sufficient for analyses of population trends. Our survey of 200 hunters shows that none of those have any interest towards the species neither as food item nor as a trophy. The survey of 50 farmers who use pasturelands at the areas where the Yellow-billed Choughs occur, mentioned that they often have seen these medium sized black birds and do not consider them as harmful ones, therefore they have never shot those. Among 50 surveyed rock-climbers, all mentioned that they have seen some black birds at the rocks but cannot say for sure whether those belong to Red-billed or Yellow-billed Choughs. Among 30 questioned cavers, seven mentioned that they have been climbing in a cave in north-eastern Armenia, where some medium-sized crow-like birds are nesting (by their opinion and not by direct evidence). They also mentioned that the cave is included now in the extreme tourism routes. The interview with the State Inspectorate for Nature Protection and Mineral Resources, shows that they have no information about the species, its rarity level, its conservation status, and have no existing information about necessary measures for its protection, except the point stated in the governmental decree (Parliament of RA 2017) about punishment for illegal shooting or trapping of Yellow-billed Chough.

Discussion

The new records expand the known distribution range of the species to the central and northern regions of Armenia. The population of the species in Armenia is estimated as a minimum of 68 adult individuals, although it appears that this number might be increased with further investigations of the North-eastern regions of Armenia, which contain a number of such underground caves and cliffs with the niches, caves and grottos. At the current level of species' understanding, the species appears to be less disturbed. Based on the obtained knowledge the species' conservation status should remain the same – Endangered, although the criteria should be modified into B2*a* – area of occupancy is less than 500 km^2 and number of locations is not more than five and *D* – number of mature individuals is less than 500 (IUCN Standards and Petitions Committee 2019). The current information is not enough to compute the population trend, so the criteria on decline are not applicable.

The Yellow-billed Choughs' distribution overlaps with the Zangezur Biosphere Complex and Yeghegis State Sanctuary, as well as with Zan-

gezur, Aragats Alpine, and Idjevan Emerald Sites protected under Bern Convention (FAYVUSH et al. 2016); however, some populations remain outside the national and international protected areas. Therefore, the section regarding conservation measures in the current Red Book of the Animals of Armenia (*The Red Book…* 2010), which states that there is no need for special protection measures also should be revised.

Taking the aforementioned into consideration, for further protection of the species, it is necessary to:

a) identify all other colonies of the species in Armenia;

b) include all colonies into Emerald Network;

c) develop a targeted educational program for rock-climbers and cavers aimed at temporal modification of their climbing and caving routs, in order to minimize disturbance to the species' in nesting season. These conservation measures should be supported by continuous monitoring of the species with two purposes: (i) to identify its population trend, and (ii) to indicate the efficiency of undertaken conservation measures.

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EFFECT OF TRIAZOLE FUNGICIDES ON FATTY ACID CONTENT IN *EISENIA FETIDA**

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Key words: ecotoxicity, fungicides, triazoles, *Eisenia fetida,* fatty acids.

Abstract

In a laboratory experiment using an artificial substrate, the toxic effects on earthworm *Eisenia fetida* of two fungicides – triazole derivatives containing tebuconazole individually and in binary combination with triadimefon was investigated. Moderate toxicity of the fungicides was determined for *Eisenia fetida*. The fatty acid (FA) profile of *Eisenia fetida* lipids was studied by high-sensitivity gas chromatography under the acute influence of fungicides at a dose, which corresponded to LC_{50}. A similar redistribution in the content of saturated and unsaturated FAs has been shown for both fungicides. The total content of saturated FAs decreased. The total content of polyunsaturated FAs increased, especially of the ω6 family, which prevails over ω3 in earthworms. The observed changes in the FAs profile of the earthworm lipids are thought to indicate their involvement in the early metabolic response under fungicidal load.

Introduction

The chemicalization of agriculture poses a serious potential danger to both agrobiocenosis and the environment, given that the number and diversity of pesticides is steadily increasing. Extremely dangerous is the impact on the soil biota, which can result in the deterioration of the soil

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ecosystem functioning (CHAGNON et al. 2015). Typical representatives of soil biota are earthworms – key organisms that ensure the decomposition of dead plants and animal remains on organic and inorganic components, which contributes to soil fertility (YE et al. 2016). Since soil organisms are prey for animals such as shrews and birds, toxicants may reach higher trophic levels.

Earthworms are sensitive indicators of changes in the ecological status of the habitat considering their environmental significance, high soil biomass and sensitivity to relatively low concentrations of pollutants (RICH et al. 2015). Laboratory experiments revealed a negative effect of pesticides on the activity of earthworms (WANG et al. 2012).

Although the earthworm *E. fetida* has been used in many ecotoxicological studies in recent years, most of them focused solely on assessing the effects of individual pesticides (RICO et al. 2016, CANG et al. 2017). It is important to study the toxic effect of pesticide mixtures on biota, considering the increasing use in agriculture of combined pesticides containing several active substances since it is difficult to predict the combined effect of pesticides due to the possibility of complex synergistic and antagonistic reactions (WANG et al. 2012, YANG et al. 2017).

The toxic effects of pesticides on biota are due to the processes of absorption and metabolism of substances in the organism (ANDERSON and ZHU 2004). An important indicator that can characterize the effect of pesticides on the organism is the qualitative and quantitative composition of lipid fatty acids (FAs), which is determined by the course of biochemical processes. In particular, the modification of the FAs composition of the honey bee *A. mellifera* lipids has been shown under the pesticide influence (KHYZHNYAK et al. 2018).

The aim of this work is to evaluate the toxic effect of fungicides – triazole derivatives containing tebuconazole in an individual and in binary combination with triadimefon – on *Eisenia fetida* in the study of the fatty acid profile of earthworm lipids.

Material and Methods

Characteristics of Fungicides

In our research we studied fungicides used on crops in Ukraine, the stock solutions of which contain the active ingredients (a.i.): AC (a.i.: tebuconazole, 250 g/dm^3) and two component AZ (a.i.: tebuconazole, 125 g/dm^3 + triadimefon, 100 g/dm^3). The active ingredients of fungicides

belong to the chemical class of triazoles (LEWIS et al. 2016). Fungicides are used in the formulation of an emulsifiable concentrate.

Acute Toxicity

In laboratory conditions (using artificial substrate) acute toxicity of fungicides (LC_{50}, 14 days) was determined by biotesting according to (OECD (1984), *Test No. 207*).

The test object for the study was the sexually mature adult individuals of the earthworm *Eisenia fetida* weighing from 300 to 500 mg. Artificial substrate with a total mass of 500 g of the following composition: sphagnum peat – 10%; kaolin clay – 20%; quartz sand – 70% of the content (by dry weight) was placed in glass containers with a capacity of 2 dm^3, thoroughly mixed with solutions of the preparations in appropriate amount: 100, 200, 400, 600, 800 and 1000 mg solution/kg artificial substrate. The control variant was an artificial substrate moistened with distilled water. Appropriate groups of test objects (40 individuals in each group) were formed for the experiment. Individuals of earthworm *E. fetida* were placed in the containers, closed by lids with apertures to provide aeration. During the study period, the following conditions were maintained: temperature 20 ±2°C, light intensity in the range of 400–800 lux, illuminated period 16 h: 8 h, soil moisture at a level close to 50% of its total moisture capacity.

The acute experiment lasted 14 days. After 7 and 14 days, the behavioral response and mortality were evaluated. At the end of the experiment the total biomass of the earthworms was determined and acute fungicide toxicity (LC_{50}, 14 days) was calculated.

Determination of the Tebuconazole Content in the Samples

The tebuconazole content after the end of the experiment (14 days) was determined in the samples of the bioobjects and the artificial substrate where these organisms were located. The samples were subjected to organic solvent extraction and subsequent chromatography using an Agilent Technologies 7900-MSD 5975C chromatographic-mass spectrometer with HP-5 MS 15 m × 0.25 μm ID × 0.25 μm column. Carrier gas pressure (helium) – 60.7 kPa. Quantitative determination was performed using the analytics-Chrom application by the method of the correlation with the standard by peak height (*Food of plants…* EVS-EN 15662:2008).

Bioaccumulation of Tebuconazole in the Earthworms

Fungicide bioaccumulation means an increased content in the earthworm body as a result of penetration through the skin and uptake with

contaminated soil. Fungicide bioaccumulation by biological objects is characterized by the bioaccumulation factor (BAF) as an index of chemical exchange between the environment and the body. It is defined as the ratio of the substance amount in the earthworm organism (mg/kg dry weight) to the content of the substance in the soil (mg/kg of substrate) (MORRISON 2000).

Fatty Acid Profile Determination

A certain amount of sample was transferred to a Teflon-coated tube, the samples were homogenized, followed by lipid extraction with chloroform-methanol mixture. Lipid hydrolysis and FAs methylation were performed according to (*Animal and vegetable*… ISO 12966-2:2017). FA methyl ethers were analyzed on a Trace GC Ultra gas chromatograph (USA), detector type: flame-ionizing.

Separation was performed on a high-polar capillary chromatographic column SPTM-2560 (Supelco, USA). The standard mixture of methyl esters of FAs "37 Component FAME Mix" (Supelco) was used to identify the acids. All reagents and solvents were of analytical quality (Merck Chemicals). For the quantitative evaluation of individual FAs the method of internal normalization was used and the relative FAs content was represented as a percentage of their total content. The following combinations of the FAs were calculated: ω3 FAs, ω6 FAs, total saturated fatty acids (SFAs), total unsaturated fatty acids (UFAs), total monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), SFAs/UFAs ratio and ω3/ω6 ratio.

Statistical Analysis

The half-lethal concentration (LC_{50}) of the pesticide was determined by probit-analysis (FINNEY 1952). The data were analyzed by computer software Origin 6.0 and Excel (Microsoft, USA) using Student's t-test. Differences were considered significant when $P < 0.05$.

Results

In the acute experiment (14 days) on the effect of the fungicide AC (tebuconazole) the reduction of the body mass of *E. fetida* test individuals was observed, which with agent application in the amount of 200, 400, 600, 800 and 1000 mg/kg substrate was 26.3; 46.8; 96.2; 99.6 and 100%, respectively ($P < 0.05$). The study of the behavioral response of *E. fetida* on the 7^{th} and 14^{th} day of exposure showed that at the fungicide content in

artificial substrate up to 100–200 mg/kg no changes were revealed compared to the control variant: the test objects were mobile and reacted equally to light and mechanical irritation. Using the agent in the amount of 400 mg or more per kg substrate, mobility, reaction to light and mechanical irritation of living individuals decreased on the 7^{th} and 14^{th} day compared with the control group, and for the agent in the amount of 1000 mg/kg substrate 100% death of test objects and their decomposition were observed already on the 7^{th} day (POLISHCHUK et al. 2018).

Similar studies were performed for the combined agent. In the acute experiment (14 days) on influence of the fungicide AZ (tebuconazole + triadimefon) body mass reduction of the test objects was also revealed, which under the conditions of the agent application in the amount of 200, 400, 600, 800 and 1000 mg/kg substrate was 24.7; 32.4; 86.2; 98.9 and 100%, respectively ($P < 0.05$). The study on the 7^{th} and 14^{th} day of exposure showed no changes in the *E. fetida* behavioral response at the agent content in the artificial substrate up to 100 mg/kg, compared to the control group: the test objects were mobile and equally responded to both light and mechanical irritation. When using the agent in the amount of 200–400 mg/kg substrate, the reduction of mobility and reaction to light and mechanical irritation of living individuals was revealed on the 7^{th} and 14^{th} day compared with the control variant, which deepened with the agent content of 600 mg/kg substrate. At 1000 mg/kg substrate 100% death of test objects was observed on the 7^{th} day and practically all dead *E. fetida* individuals were located on the substrate surface (POLISHCHUK et al. 2018).

The calculated half-lethal concentration (LC_{50}, 14 day period) of AC (tebuconazole) for *E. fetida* is 435 mg/kg substrate (Figure 1*a*). The calculated half-lethal concentration (LC_{50}, 14 day period) of AZ (tebuconazole + triadimefon) for *E. fetida* is 488 mg/kg substrate (Figure 1*b*). According to the IUPAC classification the studied fungicides exhibit moderate toxicity (LEWIS et al. 2016). It should be noted that the fungicide toxicity to *E. fetida* is mediated by bioaccumulation process that is estimated by intake of the tebuconazole, which is a component of both fungicides. The study showed that in accordance with the increased percentage of tebuconazole in AC in comparison with AZ the tebuconazole content in the artificial substrate also increased after addition of the fungicides. Besides, for AC with tebuconazole content twice higher than in AZ its amount in the earthworm body after the 14-day exposure (in terms of dry weight) in turn increased (Table 1). It was estimated that the BAF value of tebuconazole for *E. fetida* does not differ in single or binary combinations and may be an indicator of toxicity.

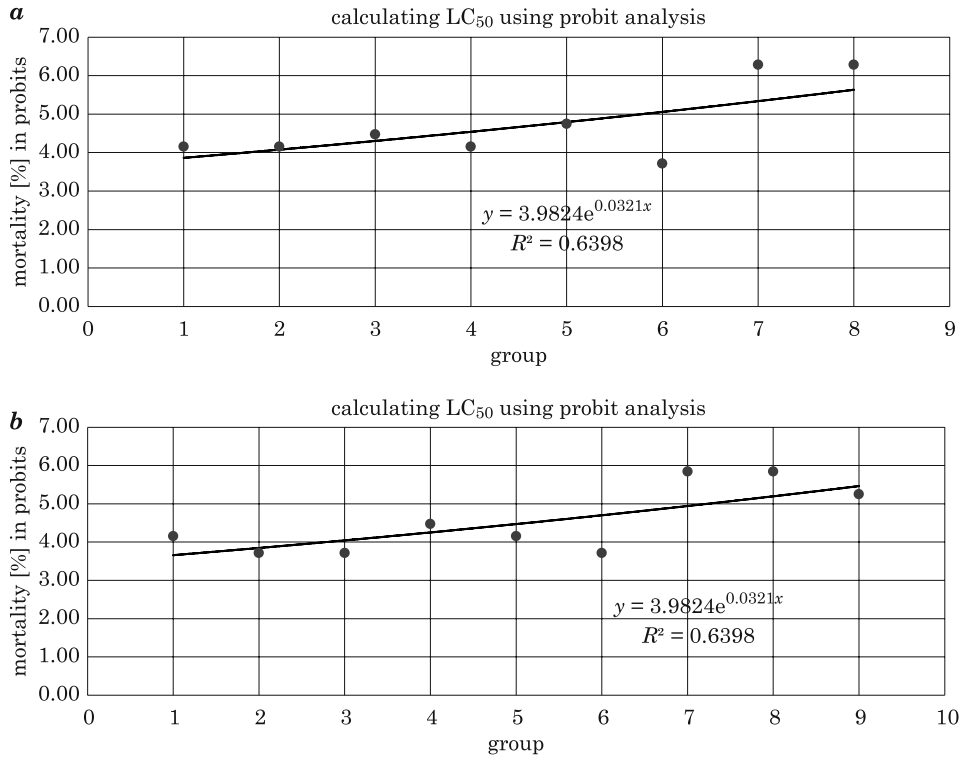

Fig. 1. Determination of half-lethal concentration (LC_{50}, 14 day period) of AC (tebuconazole) – (*a*) and of AZ (tebuconazole + triadimefon) – (*b*) for earthworms *Eisenia fetida* at 14-day exposure using probit analysis (FINNEY 1952). Note: data corresponding to Log10 concentration are given only in groups of test objects with mortality < 100%

Table 1

Indicators of tebuconazole accumulation in *Eisenia fetida* (in terms of dry weight) after 14 days of exposure

Experimental conditions	Fungicide content [mg/kg substrate]	Tebuconazole content in the substrate [mg/kg substrate]	Tebuconazole content in the body of worms [mg/kg dry weight]	Tebuconazole bioaccumulation factor (BAF)
AC (tebuconazole)	200	49.8±0.4	56.3±0.7	1.13
	400	99.6±0.5	100.3±0.9	1.01
	600	149.4±1.6	150.1±1.5	1.01
AZ (tebuconazole + triadimefon)	200	24.9±0.4	25.0±0.2	1.00
	400	49.8±0.8	57.6±0.6	1.16
	600	74.7±0.9	79.7±0.8	1.07

Note: BAF (bioaccumulation factor) – the ratio of the tebuconazole amount in the earthworm organism [mg/kg dry weight] to the content of the tebuconazole in the soil [mg/kg of substrate]

Important structural and energy components of cells, the FAs, play a significant role in the processes of metabolism, undergo reorganization at metabolic disorders and toxicant response and therefore can characterize the influence of exogenous factors on the organism of soil biota.

The high-sensitivity gas chromatography method revealed and quantified 18 FAs of earthworm *E. fetida* lipids in the control group. Saturated fatty acids (SFAs) is predominantly represented by palmitic (C16:0) and stearic (C18:0) acids. The pool of unsaturated FAs (UFAs) is represented by monounsaturated (MUFAs), in particular oleic (C18:1ω9) and polyunsaturated (PUFAs), in particular linoleic (C18:2ω6), linolenic (C18:3ω3) and arachidonic (C20:4ω6) acids (Table 2). In the conditions of the experiment no short-chain SFAs were detected in *E. fetida*. The identified profile of FAs in the control group of *E. fetida* confirms the data in the scientific literature (GUNYA et al. 2016).

The fatty acid composition of the *E. fetida* lipids after 14 days of AZ or AC exposure in the amount of 400 mg/kg substrate (close to LC_{50} value) was studied. The quantitative changes of the lipid FAs in these conditions were detected (Table 2). The total content of SFAs was reduced by 32% and 27% relative to the control ($P < 0.05$) when exposed to the fungicides AZ and AC, respectively. Of special interest is the reduction in the content of such important acids as palmitic (C16:0) and stearic (C18:0) for AZ fungicide by 22% and 31%, and for AC by 15% and 29%, respectively, compared to the control ($P < 0.05$). This may indicate a decrease of energy supply and a depletion of FAs structural reserve of earthworm organism. The saturation index (SFAs/UFAs) under the exposure to the AZ and AC decreased to 0.37 and 0.40, respectively, against 0.65 in control.

Under the conditions of an acute experiment (after 14 days) the content of lipid MUFAs, with antioxidant properties, in particular, oleic (C18:1ω9) acid, decreases. At the same time, the content of linoleic (C18:2ω6) acid slightly increases (Table 2).

Table 2

Fatty acid content [%] in *Eisenia fetida* after 14 days of exposure to the fungicides AZ (tebuconazole + triadimefon) and AC (tebuconazole) in the amount of 400 mg/kg substrate

Fatty acids [%]	Control group		AZ		AC	
	M	± *m*	M	± *m*	M	± *m*
C14:0	3.46	0.08	1.82*	0.07	1.88*	0.06
C15:0	1.27	0.04	0.22*	0.01	0.08*	0.01
C16:0	14.66	0.41	11.40*	0.50	12.52*	0.44
C16:1	0.12	0.01	0.11	0.01	0.11	0.01
C17:0	0.30	0.01	0.19*	0.01	0.23*	0.01

C18:0	17.53	0.62	12.10*	0.28	12.51*	0.28
C18:1ω9c	21.43	0.89	18.02*	0.20	17.72*	0.34
C18:2ω6c	20.43	0.43	29.28*	0.55	29.70*	0.43
C20:0	0.21	0.03	0.11*	0.01	0.16*	0.01
C18:3ω3	0.27	0.02	0.27	0.02	0.23	0.02
C20:1	2.32	0.09	0.75*	0.04	0.53*	0.02
C21:0	1.65	0.05	0.84*	0.04	1.12*	0.04
C20:2 ω6	2.10	0.09	1.31*	0.09	1.98	0.09
C22:0	0.28	0.02	0.23*	0.01	0.21*	0.01
C20:4ω6	11.23	0.23	19.32*	0.21	17.31*	0.32
C22:2ω6	1.56	0.08	2.49*	0.09	2.34*	0.16
C20:5ω3	0.66	0.03	0.79*	0.03	0.69	0.03
C22:6ω3	0.52	0.03	0.75*	0.03	0.68*	0.03
Σ SFAs	39.36±0.45		26.91±0.35*		28.71±0.41*	
Σ UFAs	60.64±0.51		73.09±0.65*		71.29±0.67*	
Σ MUFAs	23.87±0.21		18.88±0.15*		18.36±0.16*	
Σ PUFAs	36.77±0.11		54.21±0.42*		52.93±0.41*	
Σ ω3	1.45±0.04		1.81±0.04*		1.6±0.03	
Σ ω6	35.32±0.26		52.4±0.15*		51.33±0.06*	
ω3/ω6	0.04		0.03		0.03	
SFAs / UFAs	0.65		0.37		0.40	

Note: data are presented as mass fraction of fatty acid in % of total fatty acids. SFAs – saturated fatty acids, UFAs – unsaturated fatty acids, MUFAs – monounsaturated fatty acids, PUFAs – polyunsaturated fatty acids; * – $P < 0.05$ vs control

The total PUFAs content increased by 47% and 44%, respectively, for the fungicides AZ or AC in comparison with control (Table 2). Acids of the ω3 and ω6 families, the precursors of biologically active substances, are important among PUFAs (CALDER 2001). The revealed predominance of the ω6 FAs in *E. fetida* is confirmed by other findings (GRDISA et al. 2013). In experimental conditions the total content of ω6 in *E. fetida* increases which can characterize the type of eicosanoids synthesized under exogenous exposure.

Among long-chain FAs arachidonic (C20:4ω6) acid predominates which content increases in earthworm lipids under influence of the fungicide AZ or AC by 72% and 54% ($P < 0.05$) as compared to control group. However, the accumulation of arachidonic acid to ensure the vital functions of the organism in extreme conditions can lead to an increase in the content of prostaglandins in the cells and can have a negative effect. In this case, the involvement of docosahexaenoic acid (C22:6ω3) is being considered as one

of the adaptive mechanisms to regulate this process. It was found that in the conditions of the acute experiment the content of this acid in *E. fetida* increased by 44 and 31% ($P < 0.05$) for AZ or AC, respectively, compared to the control. However, in both control and experimental conditions the proportion of this acid does not exceed 1% of the total FAs (Table 2).

Studies have shown that lipid FAs of *E. fetida* are involved in the response of the body to individual and combined fungicides belonging to the chemical class of triazoles.

Discussion

Agriculture requires the use of plant protection agents to improve the crop quality and yield. However, their uncontrolled applying may entail risks to human health and the environment. The regulation of the safe use of chemicals (pesticides) requires studies of their effects on soil mesofauna. In the laboratory experiment *E. fetida* was used as the test object for the study of acute toxicity of two fungicides, triazole derivatives (in individual and binary combinations), recommended for use on crops in Ukraine. Moderate toxicity of the fungicides was determined for *E. fetida*. In particular, LC_{50} (14 days) for AC (tebuconazole) is 456 mg/kg substrate and for AZ (tebuconazole+ triadimefon) − 488 mg/kg substrate. The bioaccumulation of the fungicide in *E. fetida* under experimental conditions, which was evaluated by the BAF of tebuconazole, was shown to be similar for AC and AZ. Because parameter BAF characterizes the exchange of agents between the environment and the body, as well as their effect on metabolism, the toxic effect of triazoles was estimated by examining the FAs profile of *E. fetida* lipids considering their significant role in metabolic processes. The FAs profile of *E. fetida* lipids was analyzed using fungicides at amount of 400 mg/kg substrate corresponding to LC_{50}. Under these conditions there is a decrease in mobility and response to light and mechanical irritation of *E. fetida* individuals as well as body mass reduction by 32–46% compared to control. The obtained results indicate the same qualitative composition of FAs in the control and experimental groups of *E. fetida*. At the same time, a similar modification is observed in the quantitative content of FAs using the fungicides – triazole derivatives containing tebuconazole individually and in binary combination. In particular, the redistribution of the content of SFAs and UFAs was detected. The decrease in the level of SFAs, including palmitic (C16:0) and stearic (C18:0) acids, indicates a reduction of energy and structural component of the earthworm organism under the influence of fungicides. At the same time, the

growth of UFAs is due to PUFAs as the content of lipid MUFAs is slightly reduced. An increase in the content of UFAs can produce membrane-acting effect leading to the formation of the continuous membrane structural regions enriched with unsaturated bonds and different in charge. The change of the unsaturation degree of phospholipid FAs can contribute to the adaptation of the cellular membranes to the influence of exogenous factors (CHEBOTAREVA et al. 2011).

In view of the direct involvement of PUFAs in the regulation of most cellular processes the increase in their total content, in particular the ω6 family, can also be considered as the mobilization of adaptive body response. On the other hand, the accumulation of arachidonic acid (C20:4ω6) in the organism, which can lead to an increase in prostaglandin content in cells, along with a low content of docosahexaenoic acid (C22:6ω3) may contribute to the balance of PUFAs in this condition.

The obtained results indicate complex processes of metabolism of *E. fetida* lipid FAs at acute effect of the triazole derivatives. However, studying the profile of lipid FAs may be important for characterization of the toxic effect of triazoles on the organism, especially when combined fungicides are used. It was noted (RICO et al. 2016) that biochemical indices along with histopathological changes (muscle damage, tissue swelling, endothelial degeneration and necrosis) of *E. fetida* are sensitive biomarkers for pesticide contamination monitoring and can be proposed for a comprehensive assessment of the risk of pesticide use in agroecosystems.

Conclusions

The results of the studies indicate the involvement of fatty acids of *E. fetida* lipids in the early organism reactions on acute action of fungicides, triazole derivatives, both in individual (tebuconazole) form and binary (tebuconazole + triadimefon) combination of the active ingredients. The tested fungicides are revealed to be moderately toxic. This is characterized by a similar modification in the quantitative content of lipid FAs, accompanied by a decrease in the content of saturated FAs and a redistribution in the content of unsaturated FAs. In particular, the content of monounsaturated FAs decreases. At the same time, the increase of long-chain polyunsaturated FAs content mainly of ω6 families, which are involved in the regulation of a wide range of physiological processes, was revealed. Understanding the pesticide toxic potential for soil ecosystem representatives is essential in the practical assessment of pesticide risk for biota.

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SCREENING OF CELLULOLITIC AND XYLANOLITIC FUNGAL STRAINS FOR POSSIBLE INDUSTRIAL APPLICATIONS*

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Key words: cellulases, xylanases, fungus, screening, thermostability.

Abstract

The present study surveys sixteen fungal strains for their involvement in cellulose and xylan degradation activities. Xylanase activity was tested at pH 5.0, pH 7.0 and pH 8.5. Cultures of three, five and seven day old were screened for xylanase, β-xylosidase, cellulase (CMCase, FPase) activities and for protein production. The temperature and ionic strength effects on xylanase activity were also determined. The strains **SM7**, **SM8**, **SM9, SM10, SM12** presented remarkable xylanase activity at all pH tested when xylan or wheat bran were used as carbon source at 2%. However the greatest one (60 U/ml) was showed by **SM7** strain which also offered the highest cellulase activities (CMCase: 14 U/ml and FPase: 1.45 U/ml). Xylanase to 500 mM but decreased severely after 2.5 hours at 55°C for the majority of the strains. The highest β-D-xylosidase activity (80 U/ml) was given by **SM12** strain after seven days of culture on xylan. The **SM7** and **SM8** strains were selected for their particular behavior: the most thermostable **SM7**, offers high xylanase and cellulase activities and **SM8**, shown a very low CMCase activity but a high xylanase one. The **SM7** strain can be easily used in the bioconversion of lignocellulosic biomass and in several food processing applications, while **SM8** strain acting at alkaline pH, can be effortlessly used in paper industry.

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Introduction

Cellulose, hemicelluloses and lignin are the organic wastes from agricultural residues. In plants cell walls, the hemicelluloses are situated between the lignin and the cellulose fibers underneath. Hemicelluloses mainly consist of monomers of *D*-xylose, *D*-mannose, *D*-galactose, and L-arabinose. Xylans are the main constituents of hemicelluloses and represent the second most abundant source in the world after cellulose (Wong and Maringer 1999, Jiang et al. 2010, Khandeparker et al. 2011). They are linked to lignin, cellulose and other polymers by bonds mediated by covalent and non-covalent interactions (Coughlan and Hazlewood 1993, Chandra et al. 2012). The complex xylan structure consists of a homopolymeric backbone of *β*-1,4 linked D-xylopyranose units and short chain branches of O-acetyl, *α*-L-arabinofuranosyl, and *α*-D-glucuronyl residues (Shallom and Shoham 2003, Panwar et al. 2014). This complex xylan structure requires for its total breakdown a complex enzymatic system including xylanase (endo-1,4-ß-D-xylan xylanohydrolase, EC 3.2.1.8) which attack the main chain of xylan and *β*-xylosidase (*β*-D-xyloside xylohydrolase, EC 3.2.1.37) hydrolyzing xylo-oligosaccharide into *D*-xylose residues, in addition to a variety of disbranching enzymes: *α*-L-arabinofuranosidases (EC 3.2.1.55), *α*-D-glucuronidase (EC 2 3.2.1.1 and acetyl-esterase (EC 3.1.1.6) (Biely 1985, Collins et al. 2005, Sridevi and Charya-Singara 2011). Xylanolytic enzymes are naturally produced by diverse genera and species of bacteria, yeast and fungi. The last ones are reported to be the most potent producers (Haltrich et al. 1996, Gupta et al. 2009). Xylanases are receiving increased attention because of their biotechnological potential applications (Beg et al. 2001). They are involved in bioconversion of lignocellulosic biomass to simple sugars and in production of various industrial chemicals including biofuel (Ray 2013, Panwar et al. 2014). They are also used as a food additive to improve the nutritional properties of agricultural silage and feed grains (Kim et al. 2000, Barekatain et al. 2013). On the other hand, the use of xylanases combined with cellulases is of interest in food processing (Wong et al. 1988). The use of this enzymatic couple is favorable for the clarification of fruit juices and wine (Hang and Woodams 1997) and for the improvement of the texture and stability of bread dough (Baht and Arzlewood 2001, Kavya and Patmavathi 2009). However, the most promising application of xylanases is their effectiveness in pre-bleaching Kraft paper pulp. To this end, the hydrolysis of xylan would facilitate the release of lignin from the cellulose fibers (Salles et al. 2005, Panwar et al. 2014). Thus, the use of chlorine, which is a carcinogenic and toxic bleaching agent, is reduced (Ali and Sreekrishnan 2001,

SRIDEVI and CHARYA-SINGARA 2011). Most of the industrial processes mentioned above are carried out at high temperature and alkaline pH. The investigation of novel enzymes (xylanases and cellulases) stable under these hostile conditions is very much desired (PANWAR et al. 2014). In view of this demand, we have tried to screen new local fungal strains in order to select the most efficient in the production of ligno-cellulolytic enzymes (cellulases, xylanases) and proteins.

Materials and Methods

Chemicals

Oat spelt xylan, birch wood xylan, potato dextrose agar (PDA), carboxy methyl cellulose, *p*-nitrophenyl-ß-D-xylopyranoside, filter paper, xylose and Bovine Serum Albumin were purchased from Sigma chemicals Co., USA. Local wheat bran kindly was obtained by STPA Company (Société Tunisienne de Production Alimentaire, Sfax-Tunisia).

Fungal Strain, Media and Culture Conditions

Sixten fungal strains were tested. In fact, we use 3 strains as reference: **Rut C30**, hypercellulolytic mutant of *Trichoderma reesei* (Poutanen and Puls 1988); **CL100**, *Penicillium occitanis* (JAIN et al. 1990); **CT1**, *Penicillium occitanis* mutant (HADJ-TAIEB et al. 1992) and 13 newly isolated strains from different Tunisian biotopes (**SM1**, **SM2, SM3, SM4**, **SM5**, **SM6**, **SM7, SM8**, **SM9, SM10, SM11, SM12, SM13**).

Fungal cultures were inoculated on PDA slants and incubated at 30°C (PREBEN and SORENSEN 1997). The fully sporulated slants of six day old culture were immediately used or stored at 4°C for short term preservation. For the spore inoculums preparation, sterile distilled water (5 ml) was added to the slants and the spores were dislodged into it by gentle brushing the mycelium with a sterile wire loop. The suspension from 5 Petri-dishes was recovered and filtered on cotton. It was appropriately diluted with sterile distilled water containing 0.05% Tween 80 to obtain the required spore count. The suspensions were maintained in 20% glycerol at –20°C after the determination of the concentration (GUPTA et al. 2009). A modified version of Mandels and Weber's basal medium was used (MANDELS and WEBER 1969). It was prepared as follow(g/l):$(NH_4)_2$ SO_4 (1.4), KH_2PO_4 (2), $CaCl_2$ (0.3), $MgSO_4.7H_2O$ (0.5), $NaNO_3$ (5) and trace elements [mg/l]: $FeSO_4.7H_2O$ (0.5), $ZnSO_4.4H_2O$ (1.4), $MnSO_4.H_2O$ (1.6),

$CoCl_2SO_4.6H_2O$ (2) and 0.1% (v/v) Tween-80. It was supplemented with 2% (w/v) of oat spelt xylan or wheat bran as carbon source. The pH value was adjusted to 5.0 before autoclaving for 20 min at 121°C.

The suspension (10^6 spores/ml) of each strain was cultured in 100 ml of this medium. The inoculated Erlenmeyer's flasks (500 ml) were incubated for 7 days under shaking conditions(150 rpm at 30°C). Samples (10 ml) of 3, 5 and 7 old days, were filtered on milli-pores (0.45 µm) and used for analysis.

Enzyme and Protein Assays

D-xylanase activity was measured with the optimized method described by Bailey et al.(1992). An appropriately diluted crude enzyme (0.5 ml) was incubated with 0.5 ml of 1% birch wood xylan (prepared in 50 mM buffer of sodium citrate pH 5.0 or phosphate pH 7.0 or MPSO pH 8.5) for 30 min at 55°C. The reaction was stopped by adding 3 ml of 3,5-dinitrosalicylicacid (DNS) and the test tubes were boiled (10 min) for color development and cooled rapidly The reaction mixture was diluted with 20 ml of distilled water and the absorbance was taken at 550 nm. The amount of reducing sugars liberated was quantified using xylose as standard (MILLER 1959). One unit of enzyme activity is defined as 1 micromole of xylose released/ml enzyme/min under assay conditions.

***β*-D-xylosidase activity** was carried out according to the HERR et al. method (1978) using p-nitrophenyl-ß-D-xylopyranoside as substrate dissolved in sodium acetate buffer (50 mM; pH 5.0). A suitable diluted culture filtrate (1 ml) was incubated with substrate (5 mM) for 15 min at 50°C.

Cellulase activities: endoglucanase or carboxymethyl cellulase (CMCase) and global cellulose or filter-paper-hydrolyzing (FPase) were determined by IUPAC methods (1987). CMCase and FPase were measured by estimating the reducing sugars produced respectively from 2% (w/v) CMC and 50 mg of filter paper (Whatman N°1). The reactions were carried out in 0.05 M sodium-citrate buffered at pH 4.8 and incubated at 50°C. The reaction time was 30 min for CMCase against 60 min for FPase. One unit of either enzyme activity is defined as 1 micromole of glucose released/ ml enzyme/ min under assay conditions.

Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as the standard (BRADFORD 1976).

Characterization of Xylanase Activity

Temperature effect. A suitable diluted culture filtrate was incubated in the absence of substrate for 0 min, 90 min and 150 min at 55°C and the residual activity was determined as described above.

Ionic strength effect. Phosphate buffer (pH 5.0) was used at 50, 100, 500 and 750 mM. 1 ml of reaction mixtures containing 0.9 ml of xylan at 1% in the same buffer and 0.1 ml of suitable diluted culture filtrate were incubated for 30 min at 50°C. The released reducing sugars were determined by DNS as described above.

Statistical Analysis

All the results were the average of three determinations of two separate experiments for each cultural condition. They were statistically analyzed by SAS software (Version 8) using Duncan test performed after analysis of variance (ANOVA).

Results and Discussion

Xylanase production. Sixteen filamentous fungi strains were grown in liquid culture media supplemented with oat spelt xylan or wheat bran (2%) as carbon source incubated at 30°C. At pH 5.0, pH 7.0 and pH 8.5, samples of 3, 5 and 7 old day's culture were tested for xylanase activity. As shown in Table 1, it is clear that all the studied fungi secreted varied levels of xylanase at third, fifth and seventh cultivation day. In fact, xylolytic activities were often induced by xylan as well as by the endocellulase end product action, such cellobiose (BAILEY and POUTANEN 1989). The enzyme study production suggested that maximum xylanase activity was noted after 5 days of incubation while its activity decreased on further incubation. The strains Rut C30 (45 U/ml) and SM7 (60 U/ml) were considered as the super producing ones Table 1). It was noticed that the production level and enzymatic activities depend on strains, culture time and substrate used as carbon source. These observations were similar to those described on several studies (OJUMU et al. 2003, ALAM et al. 2005, MUHAMMAD et al. 2010). The pH of the medium is the second tested parameter, which influence the xylanase secretion and activity. At pH 5.0, the xylanase activity showed for the strains RutC30, SM4, SM5, SM6 and SM11 was more than at pH 7.0 and much at pH 8.5. On the other hand, the lowest activity

observed at acidic and alkaline pH was given by the *Penicillum* species (CL100 and CT1) as well SM1 and SM2 strains. However, at all pH tested the strains SM7, SM8, SM9, SM10 and SM12 gave the same activity. Thus, SM7 strain, the highest producer of xylanase, constitutes a potential source for the selective hydrolysis of xylan residues in lignocellulosic biomass. When wheat bran was used as carbon source, the same results were observed except that the maximum production was exhibited at the seventh day of culture. Strains CL100, CT1, SM1, SM2 and SM13 showed a very low activity at pH 7.0 and pH 8.5 (data not shown). Thus, enzymes have an optimum pH showing their maximum activity. At higher or lower pH values, their activity decreases (LENHINGER 1993).

Table 1
Xylanase production by fungal strains grown on xylan as carbon source. The enzymatic activity was determined at different pH

Strains	Xylanase activity [U/ml]								
	3 days			5 days			7 days		
	pH = 5	pH = 7	pH = 8.5	pH = 5	pH = 7	pH = 8.5	pH = 5	pH = 7	pH = 8.5
RUT C30	43.83	34.65	31.42	44.98	36.58	33.25	41.25	3.18	36.57
CL100	3.15	1.24	–	3.33	1.86	–	3.35	1.05	–
CT1	1.58	–	–	1.66	1.04	–	1.57	1.13	–
SM1	5.23	3.18	1.05	6.66	3.17	1.01	6.45	4.68	1.67
SM2	2.85	1.17	–	4.17	2.14	–	4.15	3.93	–
SM3	14.17	13.24	13.16	13.33	13.25	13.10	12.21	9.65	7.23
SM4	36.49	29.72	22.83	36.65	31.68	27.87	32.57	27.21	24.54
SM5	12.86	8.47	6.11	13.33	9.38	7.59	13.11	12.29	10.64
SM6	16.47	13.29	8.49	16.66	14.35	10.37	15.57	13.46	10.08
SM7	58.85	58.23	59.14	59.98	59.87	58.29	49.18	48.23	47.25
SM8	35.50	34.95	37.14	36.65	36.45	35.79	34.84	35.10	34.75
SM9	27.17	26.98	28.03	29.99	30.13	28.88	28.23	28.14	27.79
SM10	37.65	38.02	37.54	39.98	38.75	37.87	38.02	37.64	36.89
SM11	9.86	6.27	5.01	10.00	8.23	7.12	11.54	8.55	7.65
SM12	20.32	19.98	21.04	21.66	21.57	22.21	21.99	16.85	12.56
SM13	16.12	13.05	11.23	20.00	18.45	16.94	18.58	13.54	10.25

***β*-D-xylosidase production.** The *β*-D-xylosidase production was conducted at different carbon sources such as xylan and wheat bran. All the studied fungi secrete varied levels of *β*-D-xylosidase (Figure 1). The highest activity (80 U/ml) was recorded in SM12 strain, though the lowest one

was attributed to strain SM11 (4 U/ml) when using xylan as carbon source. The enzymatic production for SM9, SM10 and SM12 strains was twice greater at seventh than at fifth incubation days (Figure 1).

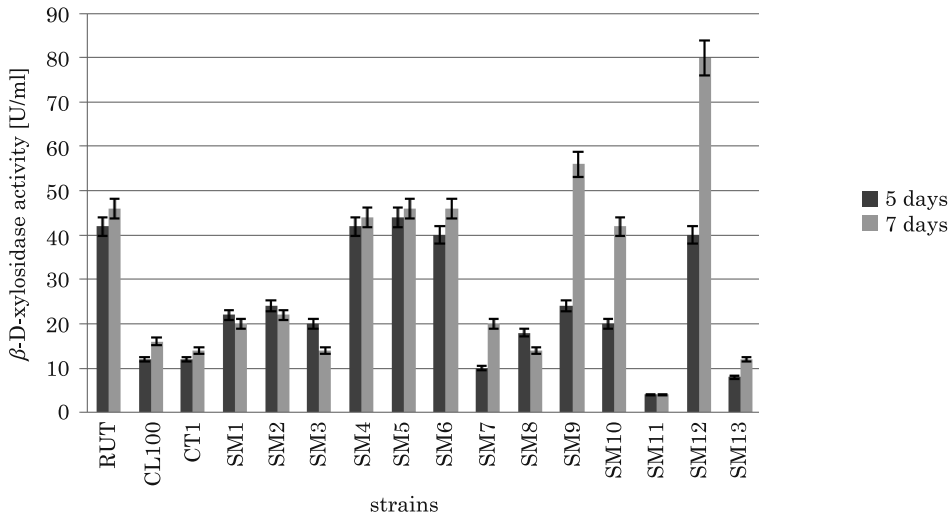

Fig. 1. *β*-D-xylosidase production by fungal strains at 5 or 7 days old culture grown on xylan as carbon source. The enzymatic activity was determined at pH 5

On wheat bran, the highest production was recorded in SM6 and SM5 strains, while the lowest one was obtained with strains SM4 and ST11 (Figure 2).

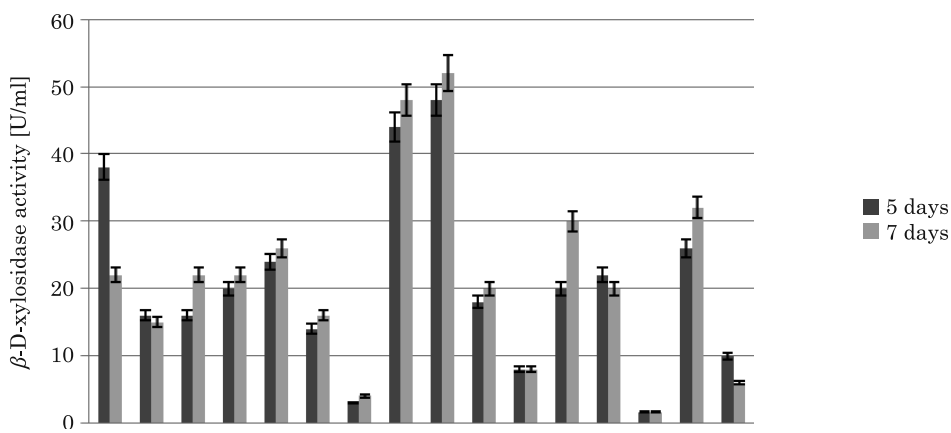

Fig. 2. *β*-D-xylosidase production by fungal strains at 5 or 7 days old culture grown on wheat bran as carbon source. The enzymatic activity was determined at pH 5

The insignificant secretion showed by SM11 strain when it's grown on xylan or on wheat bran, indicates probably a real low enzymatic production or an intracellular or a cell-bound location. Interestingly strain SM7, the highest producer of xylanase, secretes low amount of β-xylosidase indicating that the xylanase recorded is mainly due to endo type and disbranching xylanolytic enzymes. If SM7 produces considerable quantity of β-xylosidase, it would be an intra cellular or cell-bound one.

Cellulases production. Carbon source is one of the most important factors during the growth and metabolic microorganisms process. The presence of carbon sources in the cultivation medium exerted a deep effect on the enzyme production behavior of the bacterium. Among various carbon sources xylan was chosen and used to stimulate cellulase production. All the tested fungi secreted cellulase activities at varied levels (Figure 3).

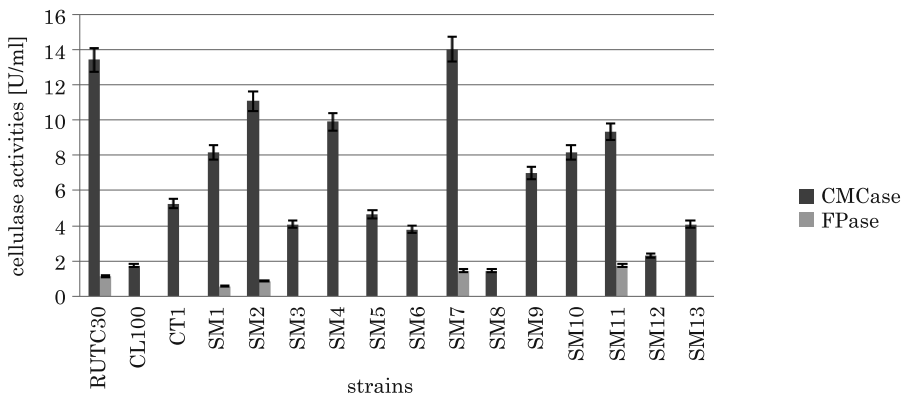

Fig. 3. Cellulases production of 5 days old culture by fungal strains grown on xylan as carbon source. The enzymatic activity was determined at pH 5

The greatest CMCase secretion was recorded in strain SM7 compared to Rut C30, the hypercellulolytic mutant of *Trichoderma reesei* producing the same amount. However, SM7 showed more important xylanase activity at alkaline conditions than Rut C30. The lowest CMCase activity was reported for SM8 and SM12 strains as well as *Penicillum occitanis* (CL100). The strain SM8 which secreted considerable xylanase and remains active at alkaline pH conditions for xylanases, acquired the property prevailing in the pulp wood (BEG et al. 2000). It is known that the use of xylanases for pulp treatment is preferable with free cellulolytic activity, since the cellulase may adversely affect the paper pulp quality and the yield (SRINIVASAN and RELE 1995, Haltrich et al. 1996). Moreover, the strain SM2 which exhibited high endoglucanase versus xylanase, can be used in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (KUHAD et al. 2011). Almost the totality

of the crude filtrates offers interesting secretion of xylanase accompanied with moderate cellulases. It is already known that the organisms secreting high level of xylanase produce simultaneously cellulase. In this context, GAMARRA et al. (2010) reported a highest yield of endoglucanase (1854 U/L) and xylanase (5051 U/L) for *Aspergillus niger* after 72 h of fermentation.

The substantial filter paper secretion (Figure 3) was given by Rut C30, SM2, SM7 and SM13 strains probably due to a high β-glucosidase or an exoglucanase activity. The increase of CMCase concentration against FPase was reported for all the strains studied and this result was confirmed by the Pandey study's (PANDEY et al. 1999).

Proteins production. Despite of the carbon source used, the protein production increased with time incubation (Figure 4 and Figure 5).

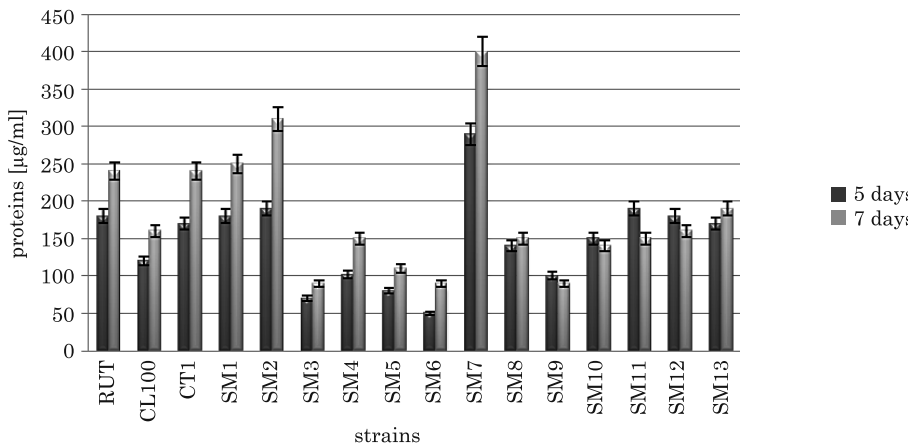

Fig. 4. Proteins production at 5 or 7 days old culture by fungal strains grown on xylan as carbon source

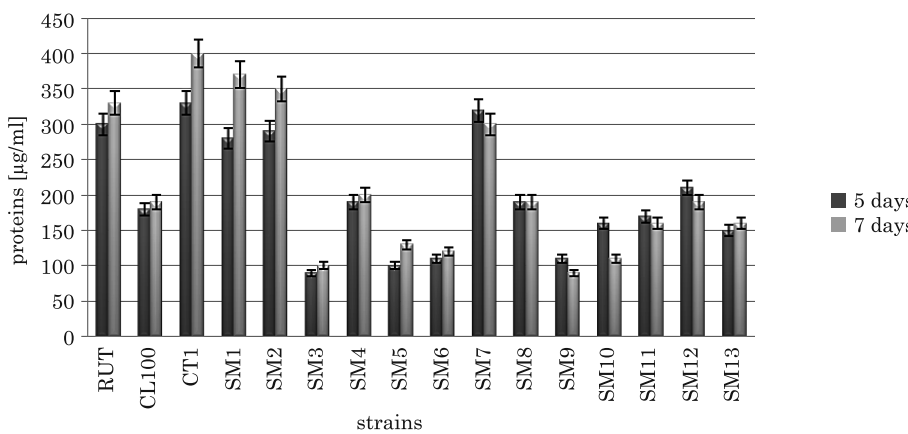

Fig. 5. Proteins production at 5 or 7 days old culture by fungal strains grown on wheat bran as carbon source

Rut C30, CT1, SM1, SM2 and SM7 strains showed the highest protein concentrations on xylan (Figure 4) or on wheat bran (Figure 5). The maximal protein level (400 μg/ml) was given by SM7 and CT1 strains cultivated respectively on xylan and wheat bran at seventh and the fifth incubation day, respectively. Thus, the protein production was correlated with the enzyme secretion.

Temperature effect on xylanase activity. The enzymatic samples were preincubated at 55°C, and their activities were measured during different incubation times. The residual activities were carried out as indicated in the enzymatic assay procedure. All the samples showed a dramatically decrease of xylanase activity after 150 min at 55°C except strains SM7, SM11 and the *Penicillium* species (CT1 and CL100) – Table 2.

Table 2

Xylanase activity characterization – thermostability at 55°C

Strains	Residual xylanase activity [%]	
	90 min	150 min
RUT C30	45	24
CL100	86	100
CT1	100	150
SM1	57	43
SM2	59	30
SM3	34	37
SM4	111	66
SM5	108	73
SM6	55	24
SM7	100	97
SM8	48	45
SM9	54	59
SM10	148	92
SM11	151	151
SM12	41	41
SM13	36	7

In fact, xylanase was reasonably stable at 55°C. Yet the remaining activities decreased very rapidly when denaturation started to take place. The maximum thermal stability was obtained at 55°C for xylanase, and no activity decrease was observed for the most strains incubated for 150 min during the first hour of incubation, displaying thereafter a constant and stable activity. Under the same conditions, significant thermal stability was unveiled by strains SM7 (97%), SM10 (92%), SM5 (73%), SM4 (66%)

and SM8 (45%) which have a better half life time than the thermostable xylanase of *Aspergillus awamori* (SOLÓRZANO et al. 2000). Furthermore, SM7 exhibited not only the highest heat stability, but also was the best xylanase and protein producer. Hence, its thermal stability can be attributed to its intrinsic protein conformation.

Ionic strength effect on xylanase activity. The ionic strength effect was tested at values ranging from 50 to 750 mM. The ionic strength variations affect the enzymatic reaction. It was shown, for all the strains that the xylanase activity was enhanced by the increase of ionic strength up to 500 mM. However, for higher concentration, enzymatic activity was not affected (Table 3).The solution ionic strength is an important parameter affecting enzyme activity. This is especially noticeable where catalysis depends on the movement of charged molecules relative to each other.

Table 3

Xylanase activity characterization – effect of ionic strength

Strains	Xylanase activity [U/ml]		
	50 mM	500 mM	750 mM
RUT C30	11.76	31.37	32.15
CL100	3.14	7.06	7.84
CT1	0	1.56	2.35
SM1	0	3.92	4.70
SM2	3.14	9.41	10.19
SM3	11.76	25.88	28.23
SM4	9.41	21.17	25.88
SM5	4.70	18.04	20.39
SM6	7.84	23.53	22.74
SM7	19.60	35.3	34.51
SM8	12.55	23.53	24.31
SM9	10.2	25.10	25.88
SM10	15.68	14.90	33.72
SM11	30.59	7.84	25.89
SM12	6.27	19.60	20.4
SM13	5.5	22.74	12.55

Thus, both the binding of charged substrates to enzymes and the movement of charged groups within the catalytic 'active' site will be influenced by the ionic composition of the medium. Even if a more complex relationship between the rate constants and the ionic strength holds, it is clearly important to control the ionic strength of solutions in parallel with the control of pH (RENKEMA et al. 2002).

Conclusion

Recent researches aim to select novel fungal strains that can produce enzymes with interesting properties and potential for future industrial applications. The present study was undertaken to explore 16 fungal strains in terms of their production of xylanase and cellulase enzymes. The screening for these enzymes-producing microorganisms, in liquid medium, resulted in the

isolation of 2 fungal strains. SM8 strain, proved to be greatly remarkable cellulase-free xylanase producing microorganisms and SM7 is the highly interesting producer of cellulose and xylanase enzymes. This could be attributed to the presence of a relatively high concentration of cellulase and /or xylanase substrates in the environment samples. These last strains were selected in order to use in the selective hydrolysis of ß 1-4 xylan residues present in lignocellulosic materials (SM7) or in paper and pulp industry (SM8). Further works are In progress to analysis enzymatic disbranching activities, and to develop suitable media and process for the enzymatic production of these selected strains.

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GENETIC HETEROGENEITY OF PORTUNID CRAB POPULATIONS FROM THREE INTERCONNECTING TOPICAL LAGOONS

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Key words: genetic variation, gene flow, polymorphism, RAPD, Crab, Lagos Lagoon, Nigeria.

Abstract

The degree of genetic variation in a population clearly specifies the structure of stocks and the probability of sustenance in future. The portunid crab, *Callinectes amnicola*, is presently managed as a single stock in Nigeria. Hence, genetic heterogeneity in this species from three interconnecting lagoons (Badagry, Lagos and Epe) was analysed using randomly amplified polymorphic DNA technique. The DNA yield and purity across populations ranged from 61.63 to 2983.34 ng/µl and from 1.68 to 1.86 respectively. The six RAPD primers: OPA-04, OPA-05, OPA-09, OPA-11, OPA-17 and OPAB-08 successfully amplified genomic DNA of 30 individual of *C. amnicola* from 3 populations with 1 region and 999 permutations. 86 RAPD fragments ranging from 96 to 1403 bp in length were generated. With 69 loci, the percentages of polymorphic bands for each primer across all populations were 60.87% (Badagry Lagoon crab), 62.32% (Lagos Harbour crab) and 66.67% (Epe Lagoon crab). Similarity index ranged from 0.848 to 0.893 and genetic distances from 0.114 to 0.165. The variations within and among the crab populations were 78% and 22% respectively. UPGMA Dendogram among *C. amnicola* populations using Nei's genetic distance obtained three main clusters, Epe Lagoon, Lagos Harbour and Badagry Lagoon, with seven outliers. The study established a relative geographical heterogeneity and limited gene flow across *C. amnicola* populations in coastal waters of Lagos, Nigeria.

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Introduction

The Lagoon crab, *Callinectes amnicola* belonging to the family Portunidae is a decapod crustacean of high commercial value in Nigeria (MORUF and LAWAL-ARE 2017). The species is generally cherished source of protein and minerals in human diet and animal feeds (CHINDAH et al. 2000, MORUF et al. 2019) and the most important food organism caught in the coastal (inshore) fishery and lagoons in West Africa (LAWAL-ARE and KUSEMIJU 2000). Portunid crab farming is well developed in Asia-Pacific region while supporting valuable commercial fisheries along the Atlantic coasts. These crabs inhabit a variety of aquatic habitats from the lower reaches of freshwater rivers, estuaries to coastal marine waters and are highly mobile, making it feasible for them to move between areas (LAWSON and OLOKO 2013).

Genetic status is essential information in fisheries management through stock enhancement or cultivation. The application of DNA markers has allowed rapid progress in aquaculture investigation of genetic variability and inbreeding, parentage assignment, species and strain identification, and the construction of high-resolution genetic linkage maps for aquaculture species (LIU and CORDES 2004). RAPD analysis uses a random oligonucleotide primer, obviating the need for knowledge of the sequences of the genome under investigation (KLINBUNGA et al. 2010); this will be useful particularly for a non-model species such as *C. amnicola* for which known nucleotide sequences of both coding and non-coding DNA in this species are rather limited. Randomly amplified polymorphic DNA (RAPD) analysis has been used to determine genetic diversity and identify useful genetic markers of various marine organisms (KLINBUNGA et al. 2007). FUJAYA et al. (2016) used RAPD markers to study the genetic variation of *Portunus pelagicus* from Makassar Straits, while SURESH and MADHURI et al. (2017) evaluated the genetic diversity of a mangrove crab of *Grapsus albolineatus*. KLINBUNGA et al. (2010) suggested that the RAPD technique is simpler and more cost-effective than amplified fragment length polymorphism (AFLP) analysis for monitoring levels of genetic diversity of *P. pelagicus*.

There is no data available regarding genetic diversity and population subdivisions of *C. amnicola* in Nigeria. This is the first report of genetic diversity of the species from Nigeria. The recognition of reproductively isolated and/or genetically differentiated populations within a species is of importance for broodstock selection and breeding programs (CONVER et al. 2006). The objectives of this study are determination of genetic diversity and intraspecific population differentiation of *C. amnicola* from three

interconnecting lagoons; Lagos, Epe and Badagry using RAPD analysis for which no data are available at present. Knowledge of the genetic diversity of *C. amnicola* in Nigeria waters is essential for the construction of an appropriate management scheme in this taxon. The basic information obtained can be applied to the construction of a genetic-based stock enhancement program for *C. amnicola*.

Materials and Methods

Study Site and Sample Collection

Three interconnecting lagoons (Badagry, Lagos and Epe) located in Southwest Nigeria were surveyed (Table 1, Figure 1). The Badagry Lagoon is part of a continuous system of lagoons and creeks along the Southwest Coast of Nigeria from the border with the Republic of Benin to the Niger Delta, with the depth of water ranging from 1–3 m and approximately 60 km long and 3 km wide (NDIMELE and KUMOLU-JOHSON 2012). The sheltered parts of sea areas where ships and boats can berth to offload and take on goods are regarded as habour. The 2 km wide Lagos harbour is geographically located at GPS co-ordinate of 6°39′16″N and 3°40′11″ E with average depth of 7.5 meters (LAWAL-ARE et al. 2018). Epe Lagoon has a surface area of 243 km^2, average depth of about 1.80 m and sandwiched between two other lagoons, the Lagos Lagoon (brackish water) to the west and Lekki Lagoon (freshwater) to the east (EDOKPAYI and IKHARO 2011).

Samples of live *Callinectes amnicola* were obtained from the catches of artisanal fishermen using traps in early hours of the day.

Table 1

Global Positioning System (GPS) coordinates of sampling locations

Locations	Latitude	Longitude
Badagry Lagoon	6°30′28″N	3°45′33″E
Lagos Harbour	6°39′16″N	3°40′11″E
Epe Lagoon	6°34′38″N	5°40′18″E

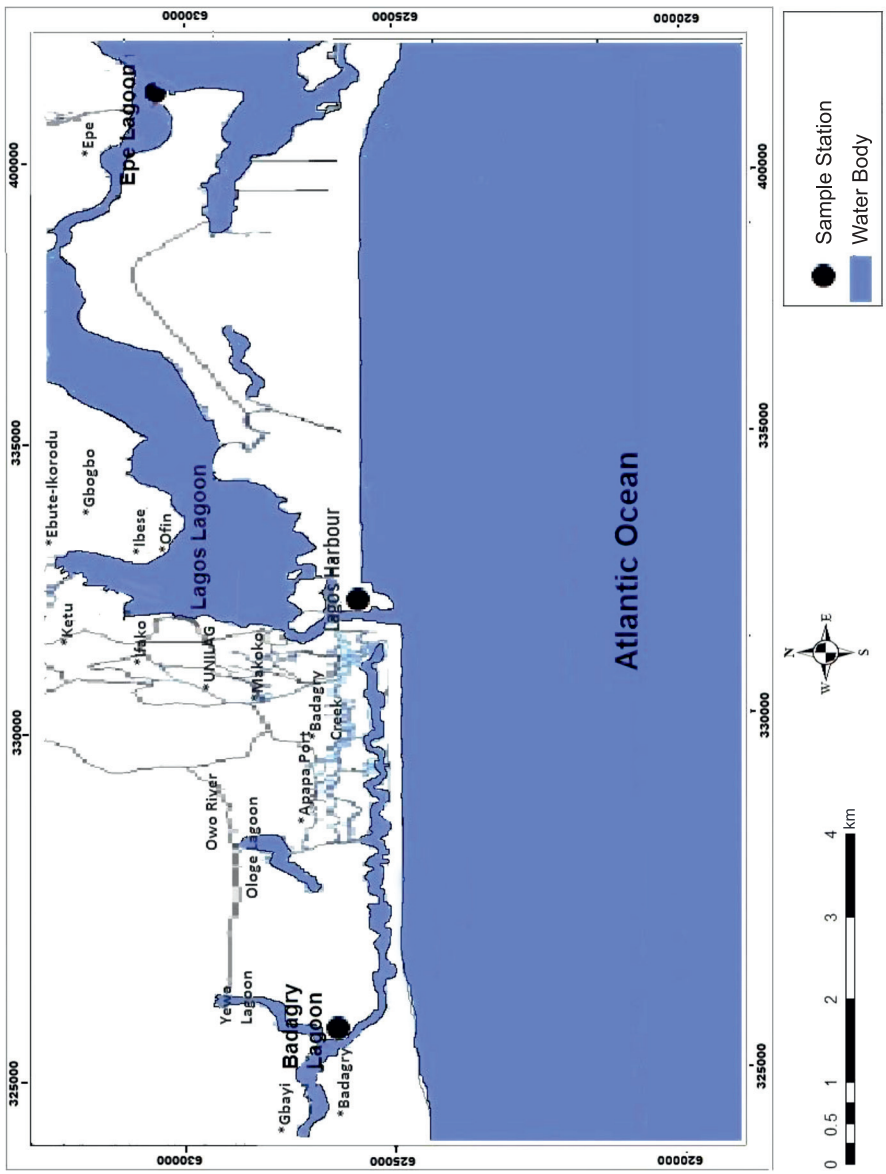

Fig. 1. Map of three interconnecting lagoons in Southwest Nigeria

Laboratory Protocol

Extraction of Genomic DNA

Genomic DNA was extracted from the muscle of the 1st periopod of each crab using a phenol-chloroform proteinase K method (KLINBUNGA et al. 1996). The concentration of the extracted DNA was spectrophotometrically estimated. DNA was stored at 4°C until needed.

Assessment of DNA Yield and Purity

DNA yield was determined with a nanodrop spectrophotometer (NANO 1000, China) based on maximum absorbance of DNA at 260 nm. 1 µL of the DNA sample was applied on the platform of the nanodrop spectrophotometer and a reading was taken after adjustment of absorbance to zero using water as blank. The yield was measured in ng/µL. The 260 nm/280 nm ratio was obtained to give an analysis of the purity of the sample and the concentration of the extracted DNA was also found.

RAPD-PCR amplification

Amplification reaction was performed in 50 ml volume mixtures consisting of Polymerase Chain Reaction buffer (50 mM KCl, 0.1% Triton X-100,10 mm Tris-HCl pH 8.3, 1.5 mM MgCl2), 2.5 mM dNTP (BioBasic, Canada), 5.0 µm of each RAPD primers, 50 ng of template DNA and 3U. Taq DNA polymerase. Six RAPD primers: OPA-04 (5' AATCGGGCTG 3'), OPA-05 (5'-AGG GGT CTT G–3'), OPA-09 (5' GGGTAACGCC 3'), OPA-11 (5'-CAA TCG CCG T–3'), OPA-17 (5'-GAC CGC TTG T–3') and OPAB-08 (5' GTGACGTAGG 3') were used in the PCR reaction. Amplifications of DNA fragments were carried out by using a thermal cycler (Hamburg, Germany) with the following cycling profile: pre-denaturation at 94°C for 4 min, followed by 35 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 36°C and 1 min extension at 72°C). The process concluded with extension at 72°C for 10 min. analysis of the resultant amplification products was done at 100 V for 4 h with 1.8% agarose gel electrophoresis (BioRAD, USA) using TBE 1 × buffer (0.9 M Tris, 0.9 M Boric acid and 20 mM EDTA, pH 8.3). Furthermore, a DNA size criterion of 100 bp molecular weight marker was used. In order to visualize the amplified products with a digital camera, ethidium bromide was used to stain them.

Agarose Gel Electrophoresis

Agarose gel (1.5 gm/100ml) was prepared in pH 8.0 buffer which contained 89 mmol of Tris-borate, 2 mmol of EDTA and 89 mmol of boric acid. After mixing the DNA samples with loading buffer, they were electropho-

resed at 50 volts for 1 hour. Afterwards, agarose gel was stained with ethidium bromide (0.5 µg/ml) for 30 minutes and then photographed on U.V light with digital camera. RAPD-PCR technique can often produce non-reproducible amplification product (CALLEJAS and OCHANDO 2002).

Data Analysis

The RAPD Polymerase Chain Reaction (PCR) banding patterns generated with the primer were analyzed using Phyllip software (version 2.1, USA). Existence or non-existence of amplicons in each lane of Agarose Gels was premised on scores recorded in binary format. Scores were exclusively allotted only to the intense and reproducible bands that ranged between 400 and 1200 bp. This was done to maintain consistency across the samples of different populations. A band that occurred was noted as "1" while the absent band was marked as "0". Parallel comparison of the amplified products in the gel with standard molecular size marker (100 bp DNA ladder) gave an estimation of molecular sizes of the RAPD products. The program was fed with the resultant data to convert the polymorphic bands into dice distance. Dendrograms were thereafter produced by the unweight pair group method using arithmetic (UPGMA) average clustering. Finally, gel Images were used to analyze banding patterns.

Results and Discussion

A better understanding of population genetic structure is important to the effective fisheries management and conservation of genetic resources in exploited marine organism (BERT et al. 2007). The six RAPD primers successfully amplified genomic DNA of 30 individual of *C. amnicola* from 3 populations (Figure 2) with 1 region and 999 permutations. 86 RAPD fragments ranging from 96 to 1403 bp in length were generated. With 69 loci, the percentages of polymorphic bands for each primer across all population samples, 60.87% (Badagary Lagoon crabs), 62.32% (Lagos Harbour crabs) and 66.67% (Epe Lagoon crabs) suggested that inbreeding is not a major concern for this economically important species. The percentage of polymorphic bands in *C. amnicola* was greater than that of the Indian mangrove crab, where the level of polymorphic bands ranged from 24.6 to 60.1% in *Grapsus albolineatus* (SURESH and MADHURI et al. 2017). Although sample size from each geographic site in this study was limited, specimens were collected from different geographic locations in Lagos Coast of Nigeria. This should be sufficient to generate the preliminary data on genetic diversity and population differentiation of *C. amnicola* in Nigeria.

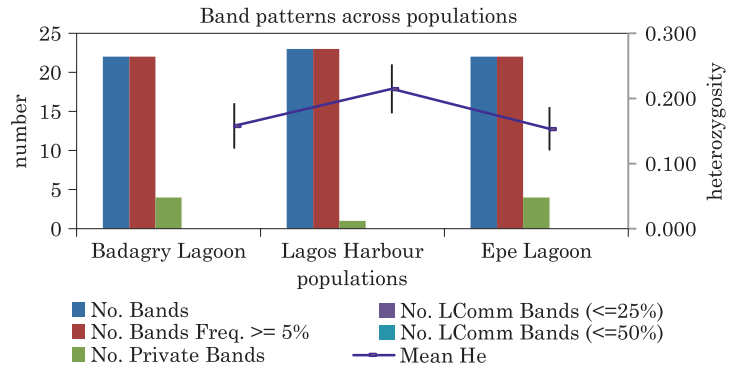

Fig. 2. Total band patterns for binary (diploid) data by populations

Theoretically, the extended planktonic larval stages of *C. amnicola* suggest high dispersal potential and the possibility of extensive gene flow between conspecific samples, at least on a geographic mesoscale of tens to hundreds of kilometers. Marine species with long larval phases are believed to have high levels of genetic variation within populations (FE´RAL 2002). According to Table 2, the large genetic distances among the geographic samples (0.114 to 0.165) indirectly reflected strong intraspecific genetic differentiation of *C. amnicola*. Generally, the levels of genetic distance between paired geographic samples did not reveal larger genetic distance with greater geographic distance (KLINBUNGA et al. 2010).

Table 2

Pairwise Population Nei Genetic Values of *C. amnicola* from three interconnecting topical lagoons in Nigeria

Population 1	Population 2	Nei Distance	Nei Identity
Badagry Lagoon	Lagos Harbour	0.140	0.870
Badagry Lagoon	Epe Lagoon	0.165	0.848
Lagos Harbour	Epe Lagoon	0.114	0.893

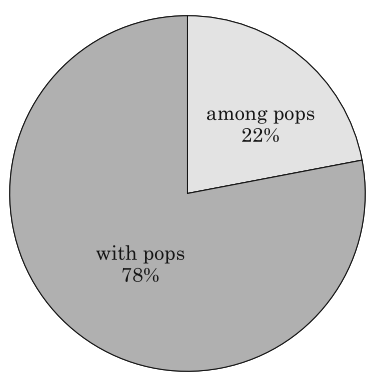

Fig. 3. Percentages of molecular variance in *Callinectes amnicola* populations from three interconnecting topical lagoons, Nigeria

The variations within and among the crab populations are 78% and 22% respectively (Figure 3) while the UPGMA Dendogram among *C. amnicola* populations using Nei's genetic distance obtained three main clusters, Epe Lagoon, Lagos Harbour and Badagry Lagoon, and seven outliers; samples 5 and 10 (Badagry Lagoon), 14 and 18 (Lagos Harbour), 21, 28 and 30 (Epe Lagoon) (Figure 4). The present study indicated that

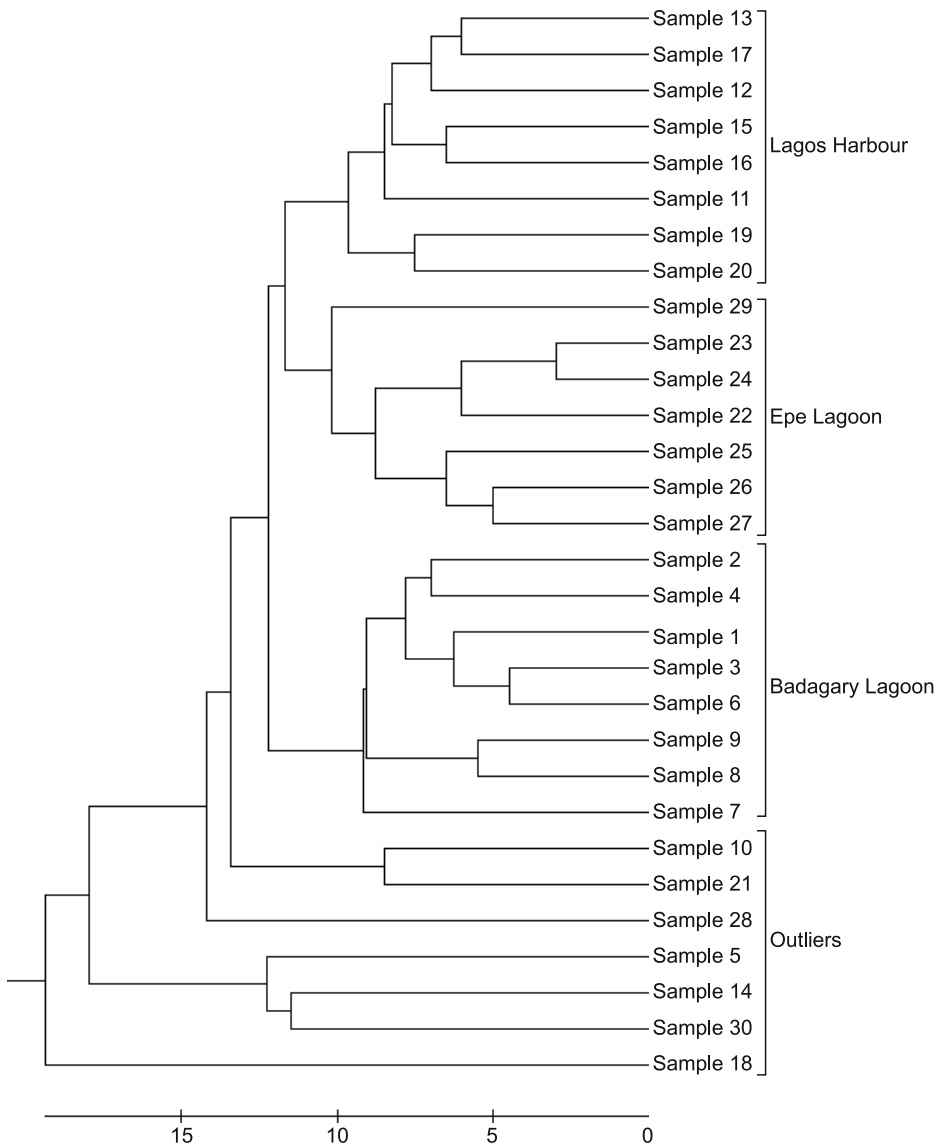

Fig. 4. UPGMA Dendrogram of three populations of *Callinectes amnicola* (De Rochecbrune 1883) using RAPD Technique

the gene pool of *C. amnicola* was not homogeneous but was microgeographically fragmented intraspecifically. Patterns of genetic differentiation at the fine-scale level in *C. amnicola* (e.g., between geographic samples approximately a few 100 km apart) were different from those of other lagoon species. For example, significant genetic heterogeneity was previously reported for the giant tiger shrimp (*P. monodon*; SUPUNGUL et al. 2000), the banana shrimp (*P. merguiensis*; HUALKASIN et al. 2003), and the abalone (*Haliotis asinina* and *H. ovina*; KLINBUNGA et al. 2003), between geographic samples from different coastal regions in Thai waters.

On the basis of the present study, three populations of *C. amnicola* were regarded as different genetic populations. From the management point of view, according to KLINBUNGA et al. (2010), these genetically isolated populations should be treated as separate management units. Stock enhancement to resolve consequent effects of overexploitation of natural *C. amnicola* may be carried out using a fine-scale level of local populations as the founders.

Conclusion

The relatively high degree of polymorphism in the population studied showed that the genetic diversity of *C. amnicola* in the Lagos coastal waters is high. High intraspecific population differentiation and restricted gene flow were observed. This information will be helpful formulating stock specific management for conservation of the species in Nigeria. In terms of aquaculture, domestication and subsequently selective breeding programs should be established for *C. amnicola* using the advantage of strong intraspecific genetic differentiation between geographically different samples of *C. amnicola* found in the present study. The proper source to be used as the founder stock for breeding programs of *C. amnicola* should be established from different genetic populations that are maintained separately. Interpopulational crosses may be carried out, possibly, to promote heterosis of economically important traits in this species. It is recommended that future studies should employ the use of newer and updated markers.

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PHYTOECDYSTEROIDS FROM *SILENE CLAVIFORMIS* AND THEIR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES

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Key words: phytoecdysteroids, *Silene claviformis,* antibacterial and antifungal activity.

Abstract

Phytoecdysteroid compounds, such as makisterone A **(1)**, polypodine B (**2**), 20-hydroxyecdysone (**3**), 2,3,20,22-diasetanide 20-hydroxyecdysone **(4)**, integristeron A (**5**), cyasterone **(6)**, 5α-2-deoxy-α-ecdysone **(7)**, *α*-ecdysone **(8)** were isolated from *Silene claviformis* plant and their structures were confirmed by NMR, ^{1}H and IR spectroscopy. In addition, an antibacterial and antifungal potential of each pure compounds and plant extracts were assessed against different microorganisms using the agar-discs diffusion assay. Results revealed that *S. claviformis* extracts and individual phytoecdysteroids did not exhibit antimicrobial activity against tested strains of microorganisms.

Introduction

The pharmaceutical industry has come to consider traditional medicine as a source for the identification of bio-active agents that can be used in the preparation of synthetic medicine. Plants are used worldwide in medicine and agriculture. Novel drugs are developed through research of

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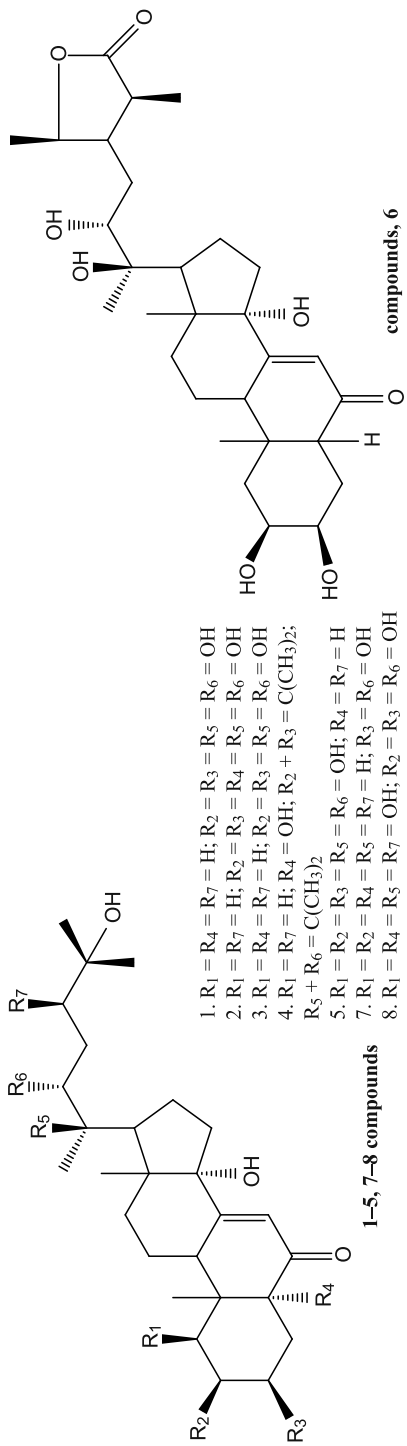
plants (USMANOV et al. 2019a, 2019b). In Uzbekistan and other parts of Central Asia, the widespread use of medicinal plants has been traditional for centuries (MAMADALIEVA et al. 2014).

Ecdysteroids represent a particularly interesting group of natural compounds from several aspects, with functions in all kingdoms of nature: in insects, they play a crucial hormonal role in controlling molting and development (KARLSON 1974); in plants, they appear to serve as part of the chemical defense against non-adapted herbivores (SCHMELZ et al. 1998). Phytoecdysteroids (ecdysteroids of plants) are a large group of polyhydroxylated steroid compounds identical or structurally similar to insects' hormones response for molting and metamorphosis (KOOLMAN 1990). The absence of the toxic action of ecdysteroids and their strong pharmacological activity served as a stimulus to search for plants producing these substances in various ecological geographic regions of Russia and abroad (MUNKHZHARGA et al. 2010). At present, more than 450 ecdysteroids have been isolated, and their structures have been established. In particular, these compounds were isolated from the plants of the *Caryophyllaceae*. The representatives of the numerous *Silene* genus *are* distinguished by the diversity of structures and the high level of ecdysteroids. *The Silene genus (Caryophyllaceae) comprises more than 700 species widely distributed in temperate zones of the world (*GOLEA et al. 2017).

There are a few reports about the antimicrobial activity of ecdysteroids. However, AHMAD et al. (1996) reported the antifungal and antibacterial activity of 20E at rather high concentrations (between 100 and 400 µg/ml, i.e. 2-8 x 10-4 M). Antimicrobial activity of 20E and its acetates was also observed by VOLODIN et al. (1999) and MAMADALIEVA et al. (2013). Toxic effects on protozoa have also been reported; rabbits receiving 20E per os (5 mg/g/day for 3 months) showed a reduced infection with *Lamblia duodenalis* (SYROV et al. 1990), and the improvement of ruminant productivity by ecdysone was also interpreted by its toxicity towards rumen protozoa (PURSER and BAKER 1994).

The present paper deals with the isolation and structure elucidation of eight PE (Figure 1) from the butanol extract of aerial parts of *Silene claviformis*, and its antimicrobial properties.



1. $R_1 = R_4 = R_7 = H$; $R_2 = R_3 = R_5 = R_6 = OH$
2. $R_1 = R_7 = H$; $R_2 = R_3 = R_4 = R_5 = R_6 = OH$
3. $R_1 = R_4 = R_7 = H$; $R_2 = R_3 = R_5 = R_6 = OH$
4. $R_1 = R_7 = H$; $R_4 = OH$; $R_2 + R_3 = C(CH_3)_2$; $R_5 + R_6 = C(CH_3)_2$
5. $R_1 = R_2 = R_3 = R_5 = R_6 = OH$; $R_4 = R_7 = H$
7. $R_1 = R_2 = R_4 = R_5 = R_7 = H$; $R_3 = R_6 = OH$
8. $R_1 = R_4 = R_5 = R_7 = OH$; $R_2 = R_3 = R_6 = OH$

Fig. 1. Structure of compounds 1–8 from *Silene claviformis*

Material and Methods

Plant Material

Silene claviformis is growing on the slopes of the lower belt of Tashkent and Samarkand district mountains. It is widespread in Central Asia. *S. claviformis* was collected in May, 2015 from the mountains Samarkand region and the plant materials were identified by Dr. Nigmatullayev A.M. at the Institute of the Chemistry of Plant Substances (ICPS), Uzbekistan. A voucher specimen (No. 2615) has been deposited in the herbarium Department of Herbal Plants in the ICPS, Tashkent, Uzbekistan.

Experimental Chemical Part

Extraction and Isolation

The freshly collected whole plant material (1 kg) was cut into small pieces and extracted three times with CH_3OH (3*5L) (each for tree days) at room temperature. The combined CH_3OH extract was evaporated under reduced pressure to yield a residue (105 g). The crude extract was suspended in water and extracted successively with $CHCl_3$, EtOAc, and BuOH. The EtOAc extract (18 g) was subjected to column chromatography (CC) on silica gel, eluting with $CHCl_3$- CH_3OH (50:1, 40:1, 30:1, 20:1, 12:1, 4:1, 2:1, 1:1), to yield four fractions. Fraction 1 (1.7 g) was further subjected to CC, eluting with CH_3OH -EtOAc (30:1), to yield compounds as Makisterone A**(1)** (0.014 g),20-hydroxyecdysone (**3**) (0.041 g). Repeated chromatography of Fraction 2 (1.5 g) over a silica gel column (CH_3OH -EtOAc, 30:1, 12:1, CH_3OH -$CHCl_3$, 4:1) yielded pure integristeron A (**5**) (0.01 g). Fraction 3 (2.1 g) was subjected to CC, eluting with CH_3OH -$CHCl_3$ (20:1), and CH_3OH -$CHCl_3$ (15:1), (12:1) to obtain 5α-2-deoxy-α-ecdysone **(7)** (0.012 g). *α*-ecdysone (0.018 g), compound **(8)** was obtained from Fraction 4 (4.3 g), which was separated through repeated column chromatography (CH_3OH - $CHCl_3$ 6:1, 4:1).

The Fraction 5 (2.1 g) was subjected to column chromatography (CC) on silica gel, eluting with $CHCl_3$- CH_3OH (100:1, 80:1, 60:1, 50:1, 30:1, 20:1, 15:1, 12:1, 9:1, 4:1, 1:1), to yield fractions which eluted with chloroform-methanol re-chromatographed and eluted with $CHCl_3$- CH_3OH increasing order of polarity. Polypodine B **(2)** (0.021 g) and 2,3,20,22-diasetonid-20-hydroxyecdysone **(4)** (0.015 g). Fraction 6 (1.2 g) was applied on a Sephadex LH-20 column with the solvent system $CHCl_3$- CH_3OH (1:1) to give compound **(6)**: cyasterone (0.035 g) from this fraction (Table 1).

Table 1

Physical and chemical properties of phytoecdysteroids isolated from *Silene claviformis*

Compound No.	Compound name	Composition	T [°C]	Yield [% of plant mass]
1	Makisterone A	$C_{28}H_{46}O_7$	263–264	0,0014%
2	Polypodine B	$C_{27}H_{44}O_8$	253–254	0.0021%
3	20-Hydroxyecdysone	$C_{27}H_{44}O_7$	242–243	0.0041%
4	20-hydroxyecdysone 2,3,20,22-diacetonide	$C_{33}H_{52}O_7$	220–221	0.0015%
5	Integristerone A	$C_{27}H_{44}O_8$	247–248	0.001%
6	Cyasterone	$C_{29}H_{44}O_8$	163–164	0.0035%
7	5α-2-Deoxy-α-ecdysone	$C_{27}H_{44}O_5$		0.0012%
8	*α*-Ecdysone	$C_{27}H_{44}O_5$	233–234	0.0018%

Experimental Biological Part

Evaluation of Antibacterial and Antifungal Activity

Test microorganisms: the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (RKMUz – 5); the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27879), *Escherichia coli* (RKMUz – 221); and the fungi *Candida albicans* (RKMUz – 247). The RKMUz microorganism cultures were obtained from the strain collection of the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan.

The antibacterial activity of extracts was determined by using the modified agar-disks diffusion method (WAYNE 2009, TASCHENBUCH 2004, ISMAILOVA et al. 2019). The bacterial cell suspension was prepared from a 24 h culture and adjusted to inoculation of $1 \cdot 10^6$ colony forming units per ml. Sterile nutrient agar (LB Agar, Invitrogen, USA, 25 g agar/l distilled water) was inoculated with bacterial cells (200 µl of bacterial cell in 2 ml 0.9% NaCl suspension and 20 ml medium) and poured into Petri dishes to give a solid medium. *Candida maltosa* ($1 \cdot 10^6$ colony forming units per ml) was inoculated into sterile Mueller-Hinton-agar according to CLSI and DIN E 58940-3 for the agar disks-diffusion assay (TASCHENBUCH 2004, ISMAILOVA et al. 2019). Forty microliters of test material (equivalent to 2 mg of the dried extract or 0.2 mg individual substances), dissolved in the same solvent used for extraction, were applied on sterile paper discs (6 mm diameter, Whatman no. 1). Ampicillin (for Gram-positive bacteria), ceftriaxone (for Gramnegative bacteria), and fluconazole

(for fungi) (Himedia Laboratories Pvt. Limited) were used as positive controls and the solvents as negative controls. The solvents were allowed to evaporate in a stream of air. The discs were deposited on the surface of inoculated agar plates. Plates were kept for 3 h in the refrigerator to enable the prediffusion of the substances into the agar. Plates with bacteria were incubated for 24 h at 37°C and plates with yeasts for 48 h at 26°C. The inhibition zone (including the disc diameter) was measured and recorded after the incubation time. An average zone of inhibition was calculated for the three replicates in independent assays.

Results and Discussion

A preliminary investigation of *S. claviformis* plant has confirmed the presence of phytoecdysteroids in its composition, and allowed to isolate and identify its main ecdysteroids (Figure 1), such as Makisterone A**(1)** (0,0014%), polypodine B (**2**) (0.0021%), 20-hydroxyecdysone (**3**) (0.0041% of dry plant's weight), (YUSUPOVA et al. 2019), 2,3,20,22-diasetonid-20-hydroxyecdysone **(4)** (0.0015%), integristeron A (**5**) (0.001%) cyasterone **(6)** (0.0035%) (YUSUPOVA et al. 2019), 5α-2-deoxy-α-ecdysone **(7)** (0.0012%), *α*-ecdysone **(8)** (0.0018%) (GIRAULT et al. 1990)

The isolated each one of ecdysteroids has been identified on the basis of the IR spectroscopy, and ^{1}H NMR spectroscopy, R_f and melting point on the A. Kruss Optronic Germany, M 5000; 90-264 VIAC, as well as by comparison with reference compounds. Table 1 provides the physicochemical data for the individual substances and ecdysteroids yield from *S. claviformis* plant. The NMR ^{1}H and ^{13}C spectra were recorded by VN MRS-400 (Varian) NMR spectrometer with an operating frequency of 400 MHz.

The *Silene claviformis* extracts were screened for their antibacterial and antifungal activities by using a modified agar diffusion method. The results of the tests showed that the extracts and individual compounds did not exhibit antibacterial and antifungal activity against tested strains of microorganisms (Table 2). This finding was consistent with the previous results, which claimed that most likely such compounds are not responsible for the antibacterial activity of the plant extracts (SHIRSHOVA et al. 2006, AHMAD et al. 1996).

Table 2

Antimicrobial effect evaluated by the diameter of inhibition zone [mm] for *Silene claviformis* plant extracts using the agar disc diffusion assay

Samples	Gram-positive bacteria		Gram-negative bacteria		Fungi
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*C. albicans*
S. claviformis ($CHCl_3$ extract) [2000 µg/disc]	na*	na	6	6	na
S. claviformis (MeOH extract) [2000 µg/disc]	6	6	6	6	na
S. claviformis (BuOH extract) [2000 µg/disc]	na	na	6	6	na
Makisterone A [2000 µg/disc]	na	na	na	na	na
Polypodine B [200 µg/disc]	na	na	na	na	na
20-Hydroxyecdysone [200 µg/disc]	na	na	na	na	na
20-hydroxyecdysone 2,3,20,22-diacetonide [200 µg/disc]	na	na	na	na	6
Integristerone A [200 µg/disc]	na	na	na	na	na
Cyasterone [200 µg/disc]	na	na	na	na	na
5α-2-Deoxy-α-ecdysone [200 µg/disc]	na	na	na	na	na
a-Ecdysone [200 µg/disc]	na	na	na	na	na
Ampicillin [10 µg/disc]	26	27	nt*	nt	nt
Ceftriaxone [30 µg/disc]	nt	nt	25	27	nt
Fluconazole [25 µg/disc]	nt	nt	nt	nt	27

* na – not active; nt – not tested

Conclusion

In this study, we revealed that the compounds of *S. claviformis* contains makisterone A (**1**), polypodine B (**2**), 20-hydroxyecdysone (**3**), 2,3,20,22-diasetanide 20-hydroxyecdysone (**4**), integristeron A (**5**), cyasterone (**6**), 5α-2-deoxy-α-ecdysone (**7**), α-ecdysone (**8**). Compounds (**1**) and (**6**) were isolated from the *Caryophyllaceae* family for the first time; compounds **(2), (4), (7)** and **(8)** were obtained for the first time from this plant. The *Silene claviformis* extracts were screened for their antibacterial and antifungal activities by using a modified agar diffusion method. The results of the tests showed that the extracts and individual compounds did not exhibit antibacterial and antifungal activity against tested strains of microorganisms.

Disclosure Statement. No potential conflict of interest was reported by the authors.

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CHEMICAL STABILITY OF INULIN IN ACIDIC ENVIRONMENT AS AN EFFECT OF A LONG-TERM STORAGE

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Key words: inulin, lactic acid, malic acid, long-term storage, model solutions.

Abstract

The aim of this study was to analyse the chemical stability of inulin in model solutions imitating a fermented milk and fruit beverage stored for 12 weeks. The two-percent (w/w) high polymerised inulin solutions were prepared and malic or lactic acid was applied to adjust pH to 3.0, 3.5, 4.0, 4.5, 5.0, before or after pasteurisation, respectively. The analysis of reducing sugars in these solutions were carried out after 1, 7, 14, 28, 56, and 84 days of storage. Inulin undergoes significant hydrolysis at pH lower than 4 in the presence of malic acid, but it is chemically stable in the presence of lactic acid during a long-term storage, which means that it can be an active prebiotic compound in dairy fermented beverages and fruit beverages with pH higher than 4.

Introduction

Inulin is a natural food ingredient found in over 36,000 plant species, among which are onion, leek, banana, chicory, garlic (NINES 1999). Due to its chemical structure and the presence of beta 1,2-glycosidic bonds, it is undigested in the human gastrointestinal system and is classified as a soluble fraction of fibre (LIGHTOWLER et al. 2018). In 2015, in order to maintain a normal defecation, the European Food Safety Authority recommended consumption of 12 g of inulin daily (EFSA 2015). Inulin also supports the development of health-promoting bacteria from *Bifidobacterium* species in the colon (MUELLER et al. 2016). Acting as a fibre and being a source of carbon for probiotic bacteria, it is applied in the production of

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fermented milk beverages (NAJAFI et al. 2019, MUZAMMIL et al. 2017). Due to relatively good solubility in water (KIM et al. 2001), it can also be used for the production of functional prebiotic beverages (DAVIM et al. 2015).

Inulin is stable at neutral and alkaline pH in water solution (GLIBOWSKI and BUKOWSKA 2011). However, in an acidic environment, inulin can be hydrolysed. The higher the acidity of the environment, the lower the chemical stability of this carbohydrate. GLIBOWSKI and BUKOWSKA (2011) showed that heating at pH 5 for one hour at 100°C did not cause hydrolysis of inulin; however, heating at the temperature of 80°C and higher for several minutes at pH 4 caused the content of reducing sugars to increase significantly in the studied solution.

Typical pH of dairy products is usually not less than 4. Furthermore, in the production process, lactic acid is produced, depending on the product, at 20–45°C (BYLUND 1995). This raises the question of how stable from chemical point of view inulin is in such products during the long-term storage. A similar question can be posed when considering the production of prebiotic drinks based on fruit juices: how will thermal processing and long-term storage in an environment with a typical pH for fruit affect the inulin?

For this reason, the aim of this study was to assess the chemical stability of inulin in model solutions imitating a fermented milk and based on fruit juice beverage stored for 12 weeks.

Materials and Methods

Materials

High polymerised inulin (Frutafit® TEX!) was kindly delivered by Sensus Operations C.V. (Roosendaal, The Netherlands). Average degree of polymerisation of inulin was ≥ 23 (manufacturer's data). Lactic acid was purchased from POCH Gliwice SA (Gliwice, Poland), while dinitrosalicylic acid, malic acid and other chemical reagents were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Preparation of Inulin Solutions

Two-percent (w/w) inulin solutions were prepared by mixing inulin with distilled water (20°C) in flasks using a MS 11HS magnetic stirrer (Wigo, Piastów, Poland). Half of the flasks was heated up to 80°C and then cooled down to 45°C in the running tap water. Subsequently, lactic acid was added in order to adjust pH of the inulin solutions to 3.0, 3.5, 4.0, 4.5,

5.0. In the other half of the flasks, the pH of inulin solutions was subsequently adjusted to 3.0, 3.5, 4.0, 4.5, 5.0 using malic acid and then the flasks were heated up to 80°C. Afterwards, the flasks cooled to ambient temperature in the running tap water. All inulin solutions were then poured into Eppendorf tubes. Inulin solutions acidified with lactic and malic acids were stored at 5 and 25°C, respectively, in a thermostatic cabinet (Pol-Eko-Aparatura, Wodzisław Śląski, Poland) for 1, 7, 14, 28, 56, and 84 days.

Determination of Reducing Sugar

The dinitrosalicylic acid (DNS) method (MILLER 1959) was used for the quantitative analysis of reducing sugar in inulin solutions. Samples were diluted with deionised water and subjected to reaction with DNS reagents. The intensity of developed colour was measured at 550 nm using a spectrophotometer (Spekol 11, Carl Zeiss Jena). Fructose (POCH, Gliwice, Poland) was used to establish a standard curve. For the analytical purpose, the reducing sugar amount was expressed as reducing sugar share in total sugar (inulin) content (GLIBOWSKI and WASKO 2008).

Statistical Analysis

The data were analysed by means of the Statistical Analysis System (SAS Enterprise Guide 3.0.3.414) using the ANOVA procedure for analysis of variance and Student-Newman-Keuls t-test for ranking the means.

Results and Discussion

Inulin does not undergo significant hydrolysis at pH 4 and higher in the presence of malic acid during a long-term storage 25°C (Figure 1). At pH below 4, significantly greater differences in reducing sugars content were found in the stored solutions up to complete hydrolysis at pH 3 after 12 weeks of storage. To meet the requirements of microbiological safety, the production technology of beverages based on fruit juices forces the addition of inulin prior to pasteurisation. For these reasons, thermal treatment of juice with pH less than 4 would mean the impossibility of producing this type of prebiotic drink. The acidity of apple, orange or mandarin juice is in the pH range of 3.35–3.85, 3.30–4.08, 3.66–3.78, respectively (AGUILAR et al. 2017, MICHALAK-MAJEWSKA et al. 2009). Other fruit juices are less acidic, e.g. grapefruit juice with pH 4.90–5.90 (AADIL et al. 2013, WANG et al. 2018) as well as vegetable juices, e.g. tomato or carrot juice,

4.4 and 6.3, respectively, (FERRARIO et al. 2017, YAN et al. 2017) which potentially could be inulin carriers. In order to confirm our results, it would be necessary to analyse the stability of inulin in beverages obtained on the basis of above mentioned juices.

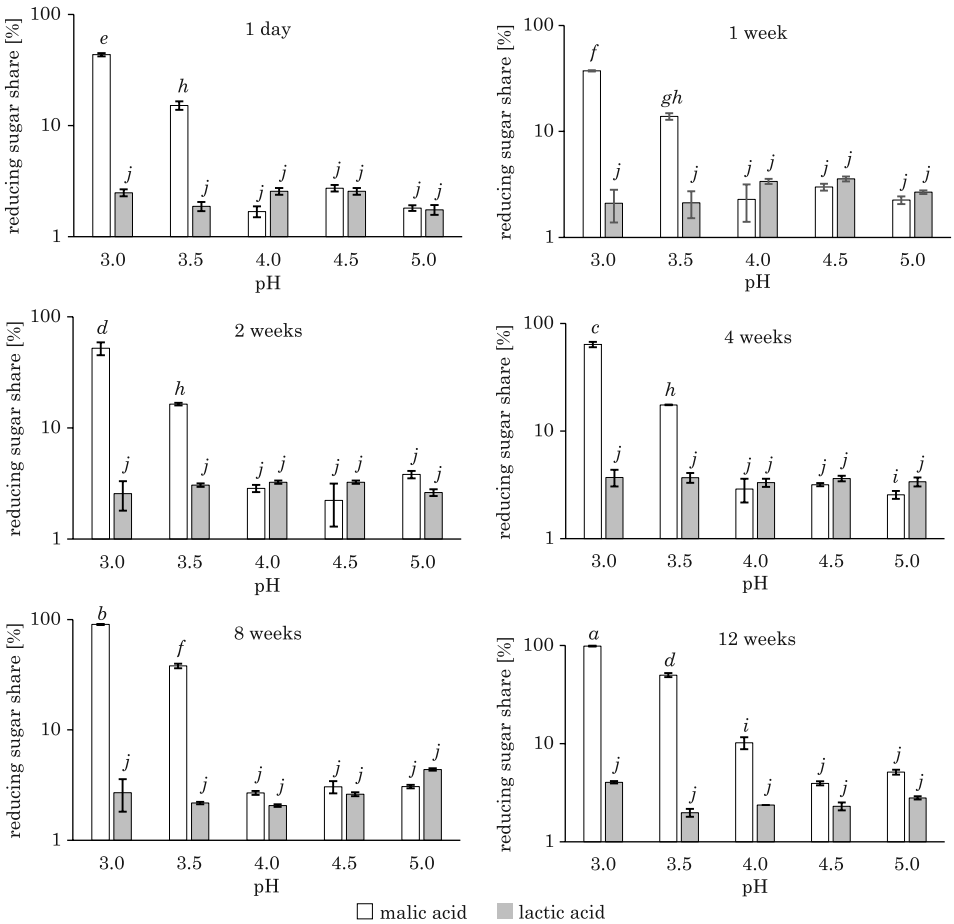

Fig. 1. Reducing sugar share (±SD) [%] in 2% inulin solutions acidified with malic or lactic acid as an effect of storage at 25 or 5°C, respectively. $^{a–j}$ Means superscripted with different alphabets differ significantly ($P \leq 0.05$)

The stability of inulin in a lactic acid solution is completely different. Regardless of the pH value and the storage time, no significant differences in the concentration of reducing sugars in the analysed solutions were found. Significant differences between malic and lactic acid result from the moment of adding the inulin to tested solutions. The experiment has been constructed to imitate the technological processes of obtaining fermented

milk beverages such as yoghurt or kefir. The first stage in the production of fermented milk beverages is pasteurisation of milk, the pH of which is almost neutral. After milk pasteurisation and cooling, the addition of microbiological cultures takes place, which converts the lactose present in milk into lactic acid. The temperature at which lactic acid is formed does not exceed 45°C, as in the case of yoghurt, and sometimes it is slightly higher than 20°C, as in the case of kefir. The production of lactic acid by lactic acid bacteria usually lasts from 5 to 24 hours, respectively in the case of yoghurt and kefir. When pH of the product is between 4 and 5, cooling and storage at the refrigeration temperature takes place (BYLUND 1995). This study shows that the refrigeration temperature does not significantly affect the hydrolysis of inulin in the analysed pH range, thus it means that there are no contraindications to the production of fermented milk beverages with the addition of inulin.

To date, many studies concerning fermented milk beverages with the addition of inulin have been carried out (DONKOR et al. 2007, GLIBOWSKI and KOWALSKA 2012, GLIBOWSKI and ZIELIŃSKA 2015, RUDRA et al. 2017). Results of these studies have shown that the presence of fructans guarantees an appropriate level of probiotic cultures (EISSA et al. 2018, GUSTAW et al. 2011) and also in the case of long-chain inulin, allows substitution of milk fat without deterioration of rheological and textural properties of final products (BRENNAN et al. 2004, GLIBOWSKI and RYBAK 2016). Until now, it was uncertain what happens with inulin in acidic milk beverage. Our research shows that inulin should not be hydrolysed due to the presence of lactic acid. A separate issue is how fructans will be used by bacteria present in this type of products, especially by probiotic cultures. In a study concerning yoghurt with inulin, COSTA et al. (2016) supposed that a part of inulin was metabolised during fermentation. This supposition support results of ZALAN et al. (2010). However, to confirm this, it would be necessary to analyse the content of inulin in dairy fermented beverages stored for a long period. However, even if a partial hydrolysis of inulin takes place in such products, it has no effect on their textural and rheological properties (GLIBOWSKI and RYBAK 2016).

Conclusions

Inulin is chemically stable in the presence of lactic acid within pH 3.0–5.0 during the long-term storage at refrigerator temperature. In case of malic acid, chemical stability of inulin during 12-week storage is unchanged when pH of the solution is higher than 4. Pasteurisation of inulin solution

acidified with malic acid to pH less than 4 followed by 84 days of storage at ambient temperature may cause its complete hydrolysis. A practical meaning of our results is that inulin can be applied as an active probiotic compound in dairy fermented beverages as well as fruit and vegetable beverages with pH higher than 4.

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BIOMETRY ANALYSIS OF EUROPEAN GRAYLING (*THYMALLUS THYMALLUS* L.) FRY, BREEDING IN RECIRCULATING AQUACULTURE SYSTEM (RAS)*

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Key words: aquaculture, RAS, morphological variation, biometry, grayling.

Abstract

The research objective has been to achieve biometric characterisation of the European grayling (*Thymallus thymallus* L.), aged 0+, cultured in the recirculating aquaculture system (RAS). The obtained results were referred to the biometric data relating to wild graylings published in literature. The body shape variation of grayling fry breeding under RAS conditions, coincides with body shape variation of the analysed wild populations in Poland.

The closest similarity to graylings from the Odra River basin was demonstrated in: the lateral length of the head, preanal length, length of pectoral fins, predorsal length, and from the Vistula River drainage basin in: preanal length, body height, distance between the pectoral and ventral fins, length of ventral fins. The biggest differences between the plastic traits of the analysed fish versus graylings from the natural environment concerned: the height of the dorsal and anal fins, length of the base of the dorsal fin, length of the base of the tail, lengths of folds of the tail fin. Analysis of the measurements revealed internal variation in the biometric characteristics of European grayling. The highest variation was observed for the parameters: anal fin height, head width, maximum body height and the smallest for: caudal fin length, eye diameter and lateral head length. The analysis results, show that the breeding of European grayling fry in RAS does not affect the variability of meristic traits.

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Principal component analysis (PCA) for the linear measurements of distances revealed that the area between the anal fin base and the head has high component loadings. As a result, it was possible to quantify the co-variation in morphological measurements in fish cultured in RAS. The PCA demonstrated three principal components, which together explained 77% of the body shape variation. The first component explains 44% of the variation, and it is composed of the postdorsal lengths, head width and maximum body height, while the second component explains 19% of the variation, and comprises the size of the eye and length of the postorbital space. The third principal component explains 14% of the variation and includes only one trait – the head lateral length. It seems that based values of the traits these three components PCA, can be carried out selection work while breeding grayling in RAS.

Introduction

European grayling (*Thymallus thymallus* L.) is a typical representative of rheophilic riverine fish (MALLET et al. 2000). In general, this species occurs in all Euroasia and North America, in cool, clean and well-oxygenated mountainous and sub-mountainous rivers (KOTTELAT and FREYHOF 2007). In Poland, it dwells in some rivers and larger streams in the Pomeranian Lake District, the southern belt of uplands, foothills and mountains, and in tributaries of the Pregoła River. Moreover, it has been introduced to some rivers outside its natural occurrence area, such as the Wel, Pasłęka, Tanew and San (WITKOWSKI et al. 1984).

For a few decades now, the number of European grayling in natural habitats have been diminishing. With the high environmental requirements of this species, the changes that take place in its habitats have an adverse influence on the stability of the species' populations. Worth mentioning are anthropogenic modifications implemented in channels and valleys of rivers (PENCZAK and KRUK 2000, OVIDIO and PHILIPPART 2002, WIŚNIEWOLSKI et al. 2004) or pollution (HONKANEN et al. 2005). Furthermore, European grayling is exposed to intense angling and poaching pressure (HOLČIK 2004, AUGUSTYN and NOWAK 2014).

Seen in this light, it appears that adequately designed and consistent fish stocking efforts should be taken in parallel to the amelioration of the aquatic environment so as to sustain the presence of European grayling in our waters. Programmes designed for the active protection of this species should have a well-defined aim and include an assessment of potential and actual outcomes (COWX 1994). For each population of endangered or overfished species, or sometimes even part of such a population, a customised approach should be implemented. To reach the presumed goals and achieve maximum environmental benefits, fish populations should be managed separately (GRIMES et al. 1987). Hence, the necessity to monitor and constantly check the efficiency of applied measures, i.e. the purposefulness of stocking efforts (TUREK et al. 2010, HORKÁ et al. 2015)

Fish stocking practice ought to strive towards the creation of stable, self-reproducing populations (FRASER 2009), and should not interfere with its genetic separateness (OCALEWICZ et al. 2013, WEISS et al. 2013). Introduction to open waters the fish stocking material that does not demonstrate the characteristics of indigenous populations leaves a permanent genetic trace (DUFTNER et al. 2005), and may have a significant influence on their current status and condition. This is particularly important because populations inhabiting various river catchments can be observed to present distinct differences with respect to some features (WITKOWSKI et. al. 1984). Similar observations can be made among populations from rivers with different flow rates.

Considering the above, a species breeding system can have an effect on subsequent differences in the growth and survivability of fish, which – after stocking – may translate into their reproductive success and angling appeal. This applies to both stocking material production of endangered fish species, and in this case, grayling in open (SZMYT et. al. 2013) and recirculation water systems. Intensive development of aquaculture, including water management systems, leads to an increasing popularity of recirculating aquaculture systems (RAS) in fish rearing. It is therefore justifiable to examine the fish stocking material obtained in such systems, both in terms of their growth and health parameters as well as measurable attributes. This is particularly important at present, when identification of intraspecies individuals or flocks of species with unique morphological traits has become more efficient and enables both better management of fish stocks – including analysis of the restocking programs effectiveness – and more effective fish protection (TUREK et. al. 2010, 2018, LEPIČ et al. 2019).

Biometric traits are an essential component of fish taxonomic research. For every species, there are patterns of scales and symbols of fins, with descriptions given as numerical values of measurable traits. They are not usually constant and are assigned a certain range of variability within a species.

It has been proven experimentally that temperature is one of the main factors that affect values of the calculable features (ORSKA 1956). It has also been demonstrated that such characteristics are affected by water parameters. Changes in the numerical values of meristic traits are mostly associated with environmental conditions and emerge in early developmental stages of fish (BEACHAM and MURRAY 1986). Studies on shapes of fish bodies prove that this feature depends on numerous factors, including the technique of swimming, availability of food, or quality of the environment. Morphometric features play an important role in identification of intraspecies variation, i.e. they can be applied as a tool to distinguish

populations adapted to various environmental or fish rearing conditions (WIŚNIEWSKA 2008, PULCINI et al. 2013). Therefore, biometric research is necessary, especially in the context of stocking material production different fish species. For example KUPREN et al. (2015) carried out allometric tests on the chub juveniles. The authors analysed the morphological development and allometric growth patterns in the aspect of the potential possibility for determining the quality of chub in restocking programs. In the case of European grayling there is little research results on biometry of this species in the wild, but there is lack information about the biometry in controlled conditions, especially in RAS.

The objective of this research has been to provide characterisation of biometric traits of European grayling (*Thymallus thymallus* L.) reared up in a recirculating aquaculture system (RAS). The research hypothesis assumed no differences in the values of meristic traits between grayling fry from RAS controlled conditions in relation to fish from the wild.

Material and Methods

The material for fry rearing was obtained from the Fish Breeding Centre of the Polish Angling Association in Paliwoda. It originated from a broodstock, spawning for the first time under controlled conditions. The broodstock in Paliwoda was created on a base reproduction material obtained from wild graylings from Biała Głuchołaska river. The experimental rearing up of the European grayling fry was conducted at the Aquaculture and Ecological Engineering Centre, the Faculty of Environmental Sciences, the University of Warmia and Mazury in Olsztyn, Poland.

Fish aged 0+, of an average initial weight of 1.5 g/indiv. ± 0.56 SD and initial length (l.t.) 5.9 cm ± 0.73 SD, were kept in plastic tanks, each having the capacity of 0.32 m^3, in a recirculating aquaculture system set up on a semi-technical scale. The average input stocking density of fish in a tank was 0.94 kg m^{-3} (200 individuals). During the fish rearing, the water temperature was maintained at 16–17°C, oxygen content was within 9.4–9.9 mg O_2 dm^{-3}, and oxygen saturation was 90–95%. Fish rearing period was 156 days. Graylings were fed by Aller Futura EX and Aller Futura commercial feed in size 1.3–2.0 mm.

All fish after being caught were, immediately anaesthetized with MS-222 (300 mg dm^{-3}) and killed by brain destruction with sharp scissors, measured and weighted. This procedure is in accordance with the guidelines in annex IV of the directive European Union number 201/63/UE.

The methodology chosen for our analysis of biometric traits was the one referred to as canonical morphometry (MARCUS 1990), as it enabled us

to make references to the literature data. Literature data cited in this work concern wild grayling of similar age and size and living in rivers with similar hydrological and environmental conditions.

The research material was analysed in terms of the variation of biometric features. Individual measurements of fish (n = 100) were taken according to the procedure illustrated in Figure 1. All measurements were performed by the same person, using a digital calliper set at 0.1 mm accuracy, except for Tl, Fl and Sl where the accuracy range was 1 mm.

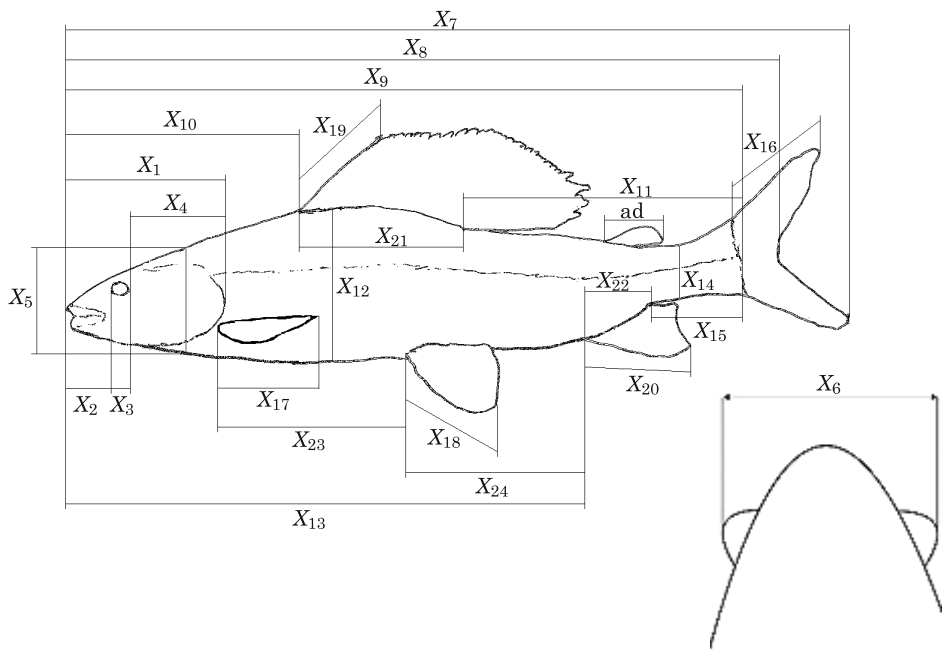

Fig. 1 Schematic design of morphometric measurements performed in the research. Description of the symbols: • shape of the head [mm]: X_1 – lc, lateral head length (*longitudo capitis lateralis*); X_2 – prO, preorbital space (*spatium praeorbitale*); X_3 – O, eye diameter (*diaemeter oculi*); X_4 – poO, postorbital space (*spatium postorbitale*); X_5 – hc, head height (*altitudo capitis*); X_6 – lac, head width (*latitude capitis*); • body shape [mm]: X_7 – Tl, total length (*longitudo totalis*); X_8 – Fl, caudal length (*longitudo caudalis*); X_9 – Sl, body length (*longitudo corporis*); X_{10} – pD, predorsal length (*longitudo praedorsale*); X_{11} – poD, postdorsal length (*longitudo postdorsale*); X_{12} – *H*, maximum body height (*altitudo corporis maxima*); X_{13} – pA, preanal length (*longitudo praeanalis*); X_{14} – *h*, minimum body height (*altitudo corporis minima*); X_{15} – lpc, length of the caudal peduncle (*longitudo peduncuil caudae*); • shape and position of fins [mm]: X_{16} – lC, caudal fin length (*longitudo pinnae C*); X_{17} – lP, pectroal fin length (*longitudo pinnae P*); X_{18} – lV, ventral fin length (*longitudo pinnae V*); X_{19} – hD, dorsal fin height (*altitudo D*); X_{20} – hA, anal fin height (*altitudo A*); X_{21} – lDbs, length of dorsal fin base (*longitudo basis D*); X_{22} – lAbs, length of anal fin base (*longitudo basis A*); X_{23} – P-V, distance between the pectoral and ventral fins (*distantia P – V*); X_{24} – *V-A*, distance between the ventral and anal fins (*distantia V – A*); ad – adipose fin length (*longitudo pinnae adiposa*)

For all measured characteristics, descriptive statistics (mean, min., max., standard deviation) were calculated and presented, alongside the variability coefficient VL of the analysed fish, which was derived from:

$$V_L = 100\ SD\ L^{-1}$$

where:
V_L – variability coefficient [%]
SD – standard deviation
L – average length (l.t.) of an individual fish [mm].

For a length-measured dimension (L), a growth-related proportional change pattern is given by the relationship between base dimension [e.g., fork length (Fl) or head length (lc)] and the L proportion (L/Fl or L/lc). The relationship between Fl (or lc) and L is allometric growth. Variation coefficient for ratio allometric was calculated following to the same formula as the total length variation coefficient. The linear measurements of distances were submitted to principal component analysis. As a result, it was possible to quantify the co-variation in morphological measurements (WIŚNIEWSKA 2008). Transformation of data was performed with the use of an allometric method postulated by ELLIOTT et al. (1995), whose purpose is to assign observed variability to differences in the body shape, not correlated to the relative size of fish. This method adjusts for residual size effects. In this paper, the method served to correct the size-dependent variability of morphometric traits, using the formula:

$$Madj = M\ (L_s/L_0)^b$$

where:
M – the original measurement
Madj – the size-adjusted value
Ls – the average value of the same trait for all fish
L_0 – is the value of a given trait
b – estimated for each trait from the observed data as the slope of the regression of log M on log L_0 using all fish in the given group.

All analyses were supported by Statistica 12.0 software.

Results

Our analysis of the measurements revealed internal variation in the biometric characteristics of European grayling (Table 1).

Table 1

Values of descriptive statistics of the analysed characteristics [mm]

MM	*M*	Min.	Max.	σ	V_L [%]
X_1	27.6	17.50	42.0	4.92	17.85
X_2	7.6	4.40	14.1	1.77	23.44
X_3	7.7	5.80	10.1	1.20	15.53
X_4	12.2	7.10	18.8	2.35	19.24
X_5	19.5	10.20	32.7	4.46	22.88
X_6	13.5	7.60	20.3	3.65	27.16
X_7	139.7	81.00	213.0	27.58	19.74
X_8	129.6	75.00	204.0	26.82	20.69
X_9	120.1	69.00	192.0	25.84	21.50
X_{10}	44.7	6.90	65.8	12.06	26.99
X_{11}	49.1	27.40	86.2	11.39	23.20
X_{12}	26.3	13.00	43.5	7.11	27.07
X_{13}	89.5	51.50	137.1	19.25	21.51
X_{14}	8.2	4.10	12.8	1.77	21.60
X_{15}	18.9	11.50	34.8	4.64	24.57
X_{16}a	18.7	11.90	24.5	2.93	15.65
X_{16}b	19.2	12.10	25.0	2.87	14.90
X_{17}	19.6	10.40	30.4	4.05	20.65
X_{18}	18.1	10.40	27.8	4.17	23.07
X_{19}	19.7	11.10	31.1	4.61	23.39
X_{20}	20.9	10.10	46.8	10.03	48.03
X_{21}	20.5	9.60	30.5	4.80	23.46
X_{22}	11.3	5.70	17.5	2.40	21.27
X_{23}	35.1	19.90	53.7	8.45	24.10
X_{24}	31.4	15.40	52.9	7.68	24.43

Explanations: MM – morphometric measurements, *M* – mean value of the trait, σ – standard deviation V_L% – variability coefficient

The lowest variation was found for the body length Sl (X_9) (V% = 1.51) and preanal length pA (X_{13}) (V% = 2.20). The highest variation was determined for the anal fin height hA (X_{20}) (V% = 29.27) and length of the dorsal fin base lDbs (X_{21}) (V% = 28.81). Relative to the caudal length Fl (X_8), the highest values were determined for the body length Sl (X_9) and preanal length pA (X_{13}). The smallest values of the proportion of a given trait to the caudal length Fl (X_8) were identified for the minimum body height *h* (X_{14}) and length of the anal fin base lAbs (X_{22}).

However, the literature on the subject there is no data regarding the above. Therefore, for reference the results to the literature data, in the subsequent step of our analysis, the data were transformed relative to the caudal length Fl (X_8) (Table 2) and lateral head length lc (X_1) (Table 3).

Table 2

Values of the plastic traits of European grayling (*Thymallus thymallus* L.) (n = 100) in relation to the caudal length *Fl* (X_8)

MM	Morphometric measurements as a % of caudal length *Fl* (X_8)				
	min.	max.	*M*	σ	V_L [%]
X_9	89.58	98.31	92.6	1.4	1.51
X_{13}	65.07	73.37	69.07	1.52	2.20
X_{10}	31.63	41.81	36.57	1.62	4.44
X_1	19.49	24.55	21.63	1.09	5.04
X_{23}	23.52	32.24	26.88	1.61	5.98
X_{18}	11.72	16.17	13.84	0.84	6.07
X_{24}	20.53	29.32	24.15	1.49	6.18
X_{17}	13.48	17.55	15.22	0.98	6.41
X_{14}	4.13	7.42	6.28	0.45	7.20
X_{22}	6.31	11.3	8.53	0.80	9.36
X_{19}	12.4	19.33	15.13	1.53	10.13
X_{15}	10.56	23.98	14.48	1.52	10.53
X_{12}	10.96	25.19	19.87	2.61	13.14
X_{21}	8.93	24.03	16.55	4.77	28.81
X_{20}	9.25	22.16	14.98	4.38	29.27

Explanations: MM – morphometric measurements; *M* – mean value of the trait; σ – standard deviation; V_L% – variability coefficient

Table 3

Values of the plastic traits of European grayling (*Thymallus thymallus* L.) (n = 100) in relation to the lateral head length *lc* (X_1)

MM	Morphometric measurements as a % of lateral head length *lc* (X_1)				
	min	max.	*M*	σ	*V* [%]
X_4	34.36	51.99	43.87	2.69	6.13
X_3	21.67	34.39	28.68	2.31	8.06
X_2	20.49	33.59	27.34	2.31	8.43
X_5	33.6	86.6	69.49	7.42	10.68

Explanations: MM – morphometric measurements; *M* – mean value of the trait; σ – standard deviation; V_L% – variability coefficient

The length of the body Sl (X_9) among the analysed European grayling specimens equalled 92.6% of the caudal length Fl (X_8). The minimum *h* (X_{14}) and the maximum *H* (X_{12}) body height of the analysed graylings was 6.28% and 19,87% of the caudal length Fl (X_8) respectively. The analysed specimens were characterised by the lateral head length lc (X_1) corresponding to 21.63% of the caudal length Fl (X_8). Predorsal length pD (X_{10}) and preanal length pA (X_{13}) of the analysed fishes equalled 36.57% and 69.07% of the caudal length Fl (X_8) respectively.

The highest value relative to the lateral head length lc (X_1) was achieved by the characteristic describing of the height head hc (X_5). The lowest approximately the same values were scored by the preorbital space prO (X_2) and eye diameter O (X_3). The highest variability coefficient values were obtained for the trait denoted as the head height hc (X_5), while the lowest one was achieved for the postorbital space poO (X_4) – Table 3.

The data transformed as explained above were submitted to a factorial analysis, in the course of which three principal components were distinguished that explained 77% of the variability of the body shape. The results of Principal Component Analysis are presented in Table 4.

Table 4

Values of the principal components and explained variation

	Component value	% of the total variance	Cumulative own value	Cumulated [%]
PC1	10.23	42.62	10.23	42.62
PC2	4.60	19.15	14.82	61.77
PC3	3.57	14.89	18.40	76.65

Contributions of principal components to explaining the variation are collated in Table 4. Loads of principal components are set in Table 5.

Table 5

Loads of principal components

Morphometric characteristics	PC2	PC2	PC3
X_1t	–	–	-0.780341
X_3t	–	0.841196	–
X_4t	–	0.802514	–
X_5t	0.71287	–	–
X_6t	0.91957	–	–
X_8t	0.87417	–	–

X_9t	0.91217	–	–
$X_{10}t$	–	0.741789	–
$X_{11}t$	0.86080	–	–
$X_{12}t$	0.90026	–	–
$X_{13}t$	0.93279	–	–
$X_{14}t$	0.77588	–	–
$X_{15}t$	0.72859	–	–
$X_{16}at$	–	0.763493	–
$X_{16}bt$	–	0.768419	–
$X_{20}t$	0.85632	–	–
$X_{23}t$	0.93550	–	–
$X_{24}t$	0.81332	–	–
Variance	**10.22789**	**4.595951**	**3.573120**
Percent	**43**	**19**	**14**

The first component explains 44%, while the second one explains 19% of the variation. The highest contribution to PC1 is made by the values of the following traits: head width lac (X_6), body length Sl (X_9), maximum body height *H* (X_{12}), preanal length pA (X_{13}), distance between the pectoral and ventral fins P-V (X_{23}). The highest contribution to the second component was assigned to the traits: eye diameter O (X_3), and postorbital space poO (X_4). This outcome proves that 44% of the variation in the body shape of domesticated European grayling arises from the postdorsal lengths poD (X_{11}), head width lac (X_6) and maximum body height *H* (X_{12}), while 19% depends on the eye diameter O (X_3) and postorbital space poO (X_4). It is interesting to note that the third component has only one contributor – lateral head length lc (X_1), yet it explains 14% of the variation. This proves the high influence of the said trait on the body shape. No contribution to the principal components is made by values of these traits: preorbital space prO (X_2), length of the dorsal fin lP (X_{17}), length of the ventral fin lV (X_{18}), height of the dorsal fin hD (X_{19}), which seems to indicate the lack of any influence of these traits on the differentiation of the body shape.

Discussion

Studies on morphological, morphometric and meristic traits, carried out to define and characterise populations, have long been performed in ichthyology. Salmonids exhibit large scale plasticity in overall body shape (CURRENS et al. 1989, BEACHAM 1990, VON CRAMON-TAUBADEL et al. 2005).

Fish populations present in areas where the water flow is rapid are typically slimmer and sleeker than those living in waters with less intensive water flow (FRANSSEN 2011, GASTON and LAUER 2015). In hatcheries, especially under RAS conditions, any phenotypic shifts will be mostly due to plasticity of traits (STRINGWELL et al. 2014). In natural conditions, in addition to the plasticity of traits the differences are probably the result of the quality of the aquatic environment. Even within a single population it is possible to identify the conditions and parameters which affect the body shape of fish (Table 5). According to PULCINI et al. (2013), compared to fish living in the wild, phenotypes of domesticated salmonid fish are characterised by a larger head, longer dorsal and anal fins and a generally less sleek body. A similar tendency in the changing body shape was detected in the course of our study on European grayling reared under controlled conditions (Table 5).

Studies conducted on several populations have shown certain differentiation of morphological traits of this species across Poland (WITKOWSKI et al. 1984). Discriminant multivariate analysis of meristic and morphometric characteristics clearly distinguished three neighbouring French populations of *T. thymallus* from the Rhone drainage basin (SURRE et al. 1986, BAJIĆ et al. 2018). In this study, multivariate analysis was used to identify the traits responsible for body shape variation, of which three main components were distinguished, which explained 77% of the observed variability (Table 4).

The length of the body Sl (X_9) among the analysed European grayling specimens equalled 92.6% of the caudal length Fl (X_8). This is less than determined in fish dwelling in two rivers from the Odra River catchment, such as the Nysa Kłodzka and the Kaczawa (93.34% and 93.37%, respectively), the Dunajec River (the Vistula River basin) (93.45%), and the Mesna River (95.38%). Approximately the same values of this proportion were found in the graylings from the Vltava River (the River Elbe tributary) and in the Danube River tributaries (91.70% and 91.44%, respectively) (WITKOWSKI and KOWALEWSKI 1979).

The lateral head length lc (X_1) of the European grayling equals 18–21% of the caudal length Fl (X_8) (WITKOWSKI et al. 1984). The analysed specimens were characterised by the lateral head length lc (X_1) corresponding to 21.63% of the caudal length Fl (X_8). This is more than the head lateral length lc (X_1) of European graylings from rivers in the Odra River basin, i.e. the Nysa Kłodzka (21.32%) and the Kaczawa (21.34%), rivers in the Danube River basin (20.83%), from the Vltava River (20.59%), the Dunajec (the Vistula River's tributary; 20.05%) or the Mesna (18.53%) (WITKOWSKI and KOWALEWSKI 1979). The result obtained in this study lies in the range

established for the Mongolian grayling (*Thymallus brevirostris* K.), in which the lateral head length lc (X_1) equals 19–24% of the caudal length Fl (X_8) (WITKOWSKI et al. 1984).

The measured predorsal length pD (X_{10}) of the analysed fishes equalled 36.57% of the caudal length Fl (X_8) – Table 2. This is higher than in European grayling caught in the Dunajec, Nysa Kłodzka, Elbe, Danube or Mesna rivers (35.43%, 35.39%, 33.94%, 35.10%, 32.51%). The European grayling caught in Kaczawa River scored a comparable value of this trait, namely 36.61% (WITKOWSKI and KOWALEWSKI 1979).

In representatives of the Mongolian grayling, this trait scores higher, at 37.5% on average, but it is lower in specimens of the Kosogol grayling (*Thymallus nigrescens* D.), between 32–34% (WITKOWSKI et al. 1984).

The preanal length pA (X_{13}) of the fish analysed was 69.07% of the caudal length Fl (X_8) – Table 2. This is less than in European grayling fish from the Mesna River (71.35%), rivers in the Danube basin (69.5%), the Dunajec River (69.55%), the Nysa Kłodzka (69.41%), or the Kaczawa (69.28%). In the fish living in the Vltava River, this trait scored lower (68.87%) (WITKOWSKI and KOWALEWSKI 1979).

The maximum body height H (X_{12}) is a trait that can reflect nutritional conditions in a water body from which given individuals originate. The maximum body height H (X_{12}) of the European grayling varied highly, from 14% to 25% of the caudal length Fl (X_8) (WITKOWSKI et al. 1984). In other representatives of the genus *Thymallus*, the value of this characteristic equals: 19–20% in the Kosogol grayling, and 15.5–24.5% in the Baikal grayling (*Thymallus baicalensis* D.) (WITKOWSKI et al. 1984). In the fish analysed in our study, the maximum body height H (X_{12}) corresponded to 19.87% of the caudal length Fl (X_8) – Table 2. Similar results were found in fish caught from the rivers: Mesna (19.68%), Dunajec (19.87%) and in the Danube River basin (20.09%). The European grayling populating the Nysa Kłodzka, Kaczawa and Vltava is distinguished by higher values of this trait (21.04%, 21.21% and 20.51%, respectively), (WITKOWSKI and KOWALEWSKI 1979). The highest values of this parameter present WITKOWSKI (1975) for graylings from Nysa Kłodzka and Kaczawa rivers – 22.56% and 22.7% respectively. Variation coefficient (V_I) for this parameter was 7.48% and 6,88% for graylings from Nysa Kłodzka and Kaczawa rivers respectively (WITKOWSKI 1975). These values are about twice lower than those obtained for graylings from RAS – 13.14% (Table 2).

The minimum body height h (X_{14}) of the analysed graylings was 6.28% of the caudal length Fl (X_8) – Table 2. This is less than found in other rivers, like the Mesna (6.65%), Veltava (7.09%), Dunajec (6.83%), Nysa Kłodzka (6.94%), Kaczawa (6.87%) or in the Danube River basin (7.12%)

(WITKOWSKI and KOWALEWSKI 1979). Similar to the previous parameter the highest values of minimum body height *h* (X_{14}) was reported by WITKOWSKI (1975) for graylings from Kaczawa river – 7.38% and Nysa Kłodzka river – 7.44% in the Odra River basin. The same author indicates the value of variation coefficient (V_L) for this parameter at the level 7.05% and 8.30% for graylings from Nysa Kłodzka and Kaczawa rivers respectively. This results there are similar to calculations related to our research – 7.20% (Table 2).

The distance between the pectoral and ventral fins P-V (X_{23}) in the analysed fish equalled 26.88% of the caudal length Fl (X_8) (Table 2) and is comparable to the value of this trait in graylings from the Vltava River (27.17%). The value of this trait determined in this study is lower than the ones obtained for fish caught in the rivers Mesna (27.98%), Dunajec (28.05%), Nysa Kłodzka (28.10%), Kaczawa (28.03%) or the rivers in the Danube River drainage basin (28.8%) (WITKOWSKI and KOWALEWSKI 1979). Variation coefficient (V_L) for X_{23} in our investigations (5.98%) was similar to WITKOWSKI (1975) publication data. The author obtained 6.13% and 5.58% of this parameter for graylings from Nysa Kłodzka and Kaczawa rivers respectively.

The space between the ventral fins and the anal *V-A* (X_{24}) fin in the analysed fish was 24.15% of the caudal length Fl (X_8) – Table 2. This is comparable to the values obtained for the fish living in the Dunajec (24.16%). Graylings in the Nysa Klodzka, Kaczawa, Vetlava and Mesna are characterised by higher values of this parameter (25.48%, 25.11%, 25.25% and 26.49%. respectively). The fish caught from rivers in the Danube River basin presented a slightly lower value of this trait (23.56%) (WITKOWSKI and KOWALEWSKI 1979).

The length of dorsal fins lDbs (X_{21}) analysed fish equalled 15.22% of the caudal length Fl (X_8) – Table 2. The value of this trait was similar in the fish originating from the Vltava River (15.32%), but lower in the grayling populations from the rivers Mesna (14.53%), Dunajec (14.58%), Nysa Kłodzka (15.01%) and Kaczawa (15.04%). The graylings living in the Danube River basin presented this trait at a higher value of 16.16% (WITKOWSKI and KOWALEWSKI 1979).

The length of ventral fins lV (X_{18}) in the analysed fish was 13.84% of the caudal length Fl (X_8) – Table 2. The closest result can be found in the fish caught from the Mesna River (14.01%), Dunajec (14.01%) and Nysa Kłodzka (14.04%). The highest value of this parameter was determined in graylings from the Kaczawa (14.54%), Vltava (15.0%) and the Danube's tributaries (15.16%) (WITKOWSKI and KOWALEWSKI 1979).

The height of the dorsal fin hD (X_{19}) in the European grayling equals 13–14% of the caudal length Fl (X_8) (WITKOWSKI et al. 1984). In the fish

measured in our study, it reached 15.13%. This is higher than in graylings from the rivers: Mesna (13.98%), Dunajec (13.05%), Nysa Kłodzka (13.95%), Kaczawa (13.93%), Vltava (13.57%), or in the catchment of the Danube (13.6%) (WITKOWSKI and KOWALEWSKI 1979).

In the fish we examined, the height of the anal fin hA (X_{20}) was 14.98% of the caudal length Fl (X_8) – Table 2. The fish from the Kaczawa River (14.47%) and the Danube River basin (14.11%) scored the closest values of this trait. Graylings from the other rivers presented lower values of this parameter, namely 11.2% in the Mesna River, 12.59% in the Dunajec, 13.3% in the Nysa Kłodzka, 13.26% in the Vltava 13.26%, (WITKOWSKI and KOWALEWSKI 1979). The length of the dorsal fin base lDbs (X_{21}) in the European grayling is 20–23% of the caudal length Fl (X_8) (WITKOWSKI et al., 1984). In the analysed grayling individuals, it equalled 16.55%. This was less than determined for graylings dwelling in the rivers Mesna (22.15%), Dunajec (20.6%), Nysa Kłodzka (22.2%), Kaczawa (21.64%), Vltava (22.25%), or in the tributaries of the Danube River (22.08%) (WITKOWSKI and KOWALEWSKI 1979).

The length of the anal fin base lAbs (X_{22}) in the analysed graylings equalled 8.53% of the caudal length Fl (X_8) – Table 2. This was less than in European grayling individuals from the rivers Mesna (9.05%), Dunajec (9.37%), Nysa Kłodzka (9.51%), Kaczawa (12.17%), Vltava (9.44%) or in rivers from the Danube River basin (9.47%) (WITKOWSKI and KOWALEWSKI 1979).

The length of the tail base lpc (X_{15}) in the analysed fish was 14.48% of the caudal length Fl (X_8) – Table 2. A similar value of this trait was found in graylings from the Vltava River (14.24%). Fish of the same species living in the Danube River basin have a slightly lower value of this parameter (14.03%). In graylings from the rivers Mesna, Dunajec, Nysa Kłodzka or Kaczawa, this trait scored higher values, i.e. 16.22%, 16.34%, 16.27%, respectively (WITKOWSKI and KOWALEWSKI 1979). The Mongolian grayling shows an even higher value of this trait, between 17–19% (WITKOWSKI et al. 1984).

The length of the upper fold of the tail fin lC (X_{16}), was around 14.63% and that of the lower fold stood at 14.96% of the caudal length Fl (X_8) – Table 2. These values are smaller than found in graylings from the rivers Mesna (16.31% the upper fold and 15.97% the lower fold), Dunajec (15.52% and 17.15%), Nysa Kłodzka (16.82% and 16.63%), Kaczawa (16.93% and 17.83%), Vltava (19.18% and 18.86%) and the Danube River basin (18.06% and 18.73%) (WITKOWSKI and KOWALEWSKI 1979).

The preorbital space prO (X_2) in the analysed graylings was around 27.34% of the lateral head length lc (X_1) – Table 3. This was less than in

graylings from the rivers Dunajec (34.48%), Nysa Kłodzka (31.47%) and Kaczawa (31.19%) according to data who was presented Witkowski et al. (1984). In other studies, similar data is given WITKOWSKI (1975) for graylings from Kaczawa river (31.07%) and Nysa Kłodzka (31.52%).

The eye diameter O (X_3) in the analysed graylings equalled 28.68% of the lateral head length lc (X_1) – Table 3. This was a higher value than determined for graylings from the Dunajec (23.78%) and Nysa Kłodzka (23.41%). Graylings inhabiting the Kaczawa River presented a similar value of the eye diameter proportion (27.79%) (WITKOWSKI et al. 1984). The postorbital space poO (X_4) in the analysed graylings was around 43.87% of the lateral head length lc (X_1). This was less than in graylings from the rivers Dunajec (47.78%), Nysa Kłodzka (48.21%) and Kaczawa (48.33%) (WITKOWSKI et al. 1984).

The head height hc (X_5) in the analysed graylings was around 69.49% of the lateral head length lc (X_1) – Table 3. The value of this trait in graylings from the rivers Dunajec (69.42%), Nysa Kłodzka (68.1%) and Kaczawa (68.91%) was similar (WITKOWSKI et al. 1984).

Breeding of European grayling fry under RAS conditions, is not only possible, but simply indicated and our results, show, that morphometric analyses can serve as a useful comparative tool in the implementation of species protection programmes. The body shape variation of grayling fry breeding under RAS conditions, coincides with body shape variation of the analysed wild populations in Poland. The closest similarity to graylings from the Odra River basin was demonstrated in: the lateral length of the head lc (X_1), preanal length pA (X_{13}), length of pectoral fins lP (X_{17}), predorsal length pD (X_{10}), and from the Vistula River drainage basin in: preanal length pA (X_{13}), body height *H* (X_{12}), distance between the pectoral and ventral fins P-V (X_{23}), length of ventral fins lV (X_{18}). The biggest differences between the plastic traits of the analysed fish versus graylings from the natural environment concerned: the height of the dorsal hD (X_{19}) and anal hA (X_{20}) fins, length of the base of the dorsal fin lDbs (X_{21}), length of the base of the tail lpc (X_{15}), lengths of folds of the tail fin lC (X_{16}).

Analysis of covariance matrix for the transformation linear measurements of distances indicated that the first three PCA explained about 77% of variance of the morphometric characters (Table 4). Based on the PCA analysis, it can be concluded that:

– 44% of breeding European grayling body shape variation arises from the postdorsal lengths poD (X_{11}), head width lac (X_6) and maximum body height *H* (X_{12}),

– 19% of the observed variation depends on the eye size O (X_3) and postorbital space length poO (X_4),

– lateral head length lc (X_1) has a big influence on the variability of the body shape, maximum body height H (X_{12}) explains 14% of the variation the body shape.

It seems that based values of the traits these three components PCA, can be carried selection work out while breeding grayling in RAS.

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LEGAL NORMS CONCERNING AQUATIC ANIMAL DISEASES IN REGULATION (EU) 2016/429 – *ANIMAL HEALTH LAW*

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Abstract

This study analysed the latest changes in European health regulations in the aquaculture sector. The following research methods were used: grammatic, systemic, teleological and pro-European interpretation of legal texts. The basic normative act subjected to analysis and interpretation was the Animal Health Law (Regulation (EU) 2016/429). Although the existence of 'codification' trends has been found in European law, it has been shown that the tools used are not adequate to the intended purposes and the works are carried out in a non-holistic manner and of a compilation nature. A shortage of normative regulations, as well as inconsistencies and non-fulfilment of obligations by EU bodies, was also indicated. This paper analysed the extent the studied area is another sphere of influence of European law on the national legal orders of the Member States, as well as the mutual relations of veterinary law and veterinary medicine concerning fish, shellfish and molluscs.

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Introduction

The Animal Health Law (AHL), i.e. the Regulation (EU) 2016/429 on transmissible animal diseases and amending and repealing certain acts in the area of animal health approved by the European Parliament and the European Council on the March 9, 2016, is one of the most significant normative acts on animal health. This AHL was published on March 31, 2016, in the Official Journal of the European Union (OJ) L 84 and entered into force on the April 21, 2016 and shall be applied from April 21, 2021.

The AHL comprises the numerous existing, yet not unified, regulations on animal health in one legal act, including the laws on the elimination of infectious diseases in animals, operation of the national animal market and animal-derived products (Terech-Majewska et al. 2011). This legal act provides a unified and simplified regulation basis; it is not only a compilation but also serves as the recodification of 39 normative acts in total, such as the directives: 64/432/EEC, 77/391/EEC, 78/52/EEC, 80/1095/EEC, 82/894/EEC, 88/407/EEC, 89/556/EEC, 90/429/EEC, 91/68/EEC, 91/666/EEC, 92/35/EEC, 92/65/EEC, 92/66/EEC, 92/118/EEC, 92/119/EC, 2000/75/EC, 2001/89/EC, 2002/60/EC, 2002/99/EC, 2003/85/EC, 2004/68/EC, 2005/94/EC, 2006/88/EC, 2008/71/EC, 2009/156/EC, 2009/158/EC; decision 95/410/EC; and regulations 1760/2000 (EC), 21/2004 (EC), and 576/2013 (EU).

The objective of the paper is an objective analysis of the Animal Health Law to improve the productivity and efficiency of the veterinary administration bodies in public health and environment protection.

Material and Methods

This study analysed the latest changes in European health regulations in the aquaculture sector, particularly the Animal Health Law (Regulation (EU) 2016/429). The analysis was carried out with legal interpretation and analysis methods, such as grammatic, systemic, teleological, purposive and pro-European interpretation.

The Subject and Objectives of the AHL

The investigated regulation establishes the laws on the prevention of infectious diseases in animals, which are transmissible or transmitted on animals or humans, as well as the laws on the eradication of these diseases. Importantly, considering the Polish veterinary nomenclature, not only is the translation of the individual sections of the legal statements

incorrect, but the title of the Act is also mistranslated: *choroby przenośne* instead of the correct name *choroby zakaźne*, or at least *choroby transmisyjne*. The mistakes and inconsistencies demonstrate the low level of the Act's legislative quality, and they present an additional challenge for the interpretation and implementation of this law.

The objective of these laws consists in the veterinary *rationes* and those related to the public health protection, such as improving the health of animals, reducing the negative effects on animal health, public health and the environment caused by the diseases included in the legislation as well as supporting sustainable aquaculture and agricultural production in the European Union. They are intended to improve the efficiency of the European internal market and address the practical demands of the environment and the resources of the aquaculture industry (ANTYCHOWICZ 2010, MALINOWSKA and BŁOŃSKA-WLAZŁOWSKA 2004, KENIG-WITKOWSKA 2011, TERECH-MAJEWSKA et al. 2011).

The implemented legal norms include the relations between the animal health and the public health and the environment, such as biological diversity; precious genetic resources; consequences of progressing climate changes; food and feed safety as well as animal welfare, such as reducing unnecessary pain, stress or suffering, antimicrobial resistance and food safety (POSYNIAK 2015). They also encompass the economic and social (legal, cultural and environmental) effects caused by the implementation of disease prevention and eradication tools (BIAŁEK et al. 2015, FELSMANN et al. 2019, SIWICKI 2020, ZWIĄZEK 2016).

Secondary and Delegated Legislation

The European Commission was obliged to approve the key delegated and secondary legislation (KENIG-WITKOWSKA 2011) to the investigated regulation within three years from the enforcement date, i.e. until March 31, 2019, to ensure that the member states have enough time for transposition (Table 1).

Table 1

Delegations of the European Commission – competence to issue acts based on the Regulation 2016/429 (UE)

Delegated acts (DA)		Implementing acts (IA)	
No. of delegations	No. of key acts (*a*)	No. of delegations	No. of key acts (*a*)
111+2 (*b*)	41	69+1 (*c*)	21

Explanations: (*a*) – so-called key acts – the European Commission has a legal obligation to issue and publish them; (*b*) – references to delegations in various articles on the basis of Art. 223, 225 of the discussed regulation; (*c*) – references to Art. 221, 225 of the discussed regulation

With the delegated acts, the Commission can complement less important elements and implement some minor changes, for example, to determine the specific tools; if the European Parliament and the European Council do not object, these acts will come into force (KENIG-WITKOWSKA 2011). The secondary legislation is used by the Commission – via the supervision of the committees composed of the EU member state representatives – to determine the conditions that will ensure unified implementation of the law throughout the European community.

To date (legislation in force as of April 1, 2020), the national implementation legislation, respective for the discussed area, has not been released.

Comparison of the Former and New Normative Arguments Relating to Aquatic Animals

The current European legislation on aquatic animals and aquaculture is based on the basic act, i.e. the European Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products, and the prevention and control of certain diseases in aquatic animals. The Directive was amended by the European Commission Directive 2008/53/EC and the Implementing Directives of the European Commission 2012/31 and 2014/22. The other important acts of European law on the discussed subject include the European Commission Decisions 2008/392, 1251/2008, and 2008/892, as well as the European Commission Regulation 1251/2008.

The norms specific for the aquaculture animals include the requirements for the marketing, import and transit of these animals and their products; minimal preventive measures; minimal tools for disease eradication; the tools to be implemented in the case of a suspected or sudden outbreak of some diseases of the aquatic animals; and the background procedures for possible outbreaks of aquatic animal diseases, such as increasing the awareness and preparedness of the public authorities, entrepreneurs and other bodies.

In the existing EU legislation, there have been individual regulations on terrestrial animal health and separate ones on the aquatic animal health (KENIG-WITKOWSKA 2011, JEDLECKA 2002). In most cases, the major rules determining the good management of animal health and good animal production practices apply to both groups of animals: terrestrial and aquatic species. This conclusion has empowered a change in European norms.

A novel approach of the European Union authorities to the health of aquatic animals and the respective regulations is not evident in the AHL. It interferes with the existing legislation on the aquatic animals and

aquaculture that was combined with the regulations referring to terrestrial animal health and other animals. The aquatic animals (at each stage of their development (so not only the adult specimens but also the eggs, sperm cells and gametes) are defined in the legal context as fish classified in the Agnatha superclass (*Agnatha*) and the Chondrichthyes class (*Chondrichthyes*), the Sacropterygii clade (*Sarcopterygii*), and the Actinopterygii class (*Actinopterygii*), aquatic molluscs are classified in the *Mollusca* phylum and aquatic crustaceans are classified in the *Crustacea* taxon. Terrestrial animals are defined in a legal context, as opposed to zoology, as birds and terrestrial mammals as well as bees and bumblebees.

Harmonisation of the European legal regulations can be achieved not only on the member-state level, i.e. via the implementation of new legal norms that are consistent with the European assumptions (different types of implementation) but also within European Union law in a broader sense (Malinowska and Błońska 2014, Kenig-Witkowska 2011). To that end, the AHL consolidates and mutually adjusts the regulations, in the specific cases, on the animal health matter of different species.

The basic normative act referring to the aquatic animals, i.e. Directive 2006/88/WE, has been included in the AHL using the compilation and correlation method (Kenig-Witkowska 2011). This directive shall expire on April 21, 2021, like the other 30 European legal acts (mainly directives, with some decisions and regulations). The references to the acts that precede the AHL should be considered as AHL cross-references, according to the correlation table included in Annex 5, item 26, concerning art. 270, section 2 of the AHL.

However, the regulations referring to the registrations and authorization of production sites and traceability and transfer of animals within the European Union have not been completely harmonised and consolidated. Unfortunately, a unified and codex act encompassing the whole legislation on animal health has not thus been developed. The approach adopted in the past is still applied for some areas, according to it, separate legal acts and individual regulations are legislated on animal health for both terrestrial and aquatic animals due to their different habitats and, therefore, specific requirements for health protection. For instance, part 4, section 2 (Art. 172–226) of the AHL applies to aquatic animals and non-aquatic animals that can transmit diseases of aquatic animals and to the animal-derived products from, or of, aquatic animals.

The delegated, secondary (Table 2) and implementing acts on the aquaculture animals should have been released by the European Commission by March 31, 2019, but this has not yet happened.

Table 2

The material scope of acts of lower rank, on the basis of the AHL

Delegated acts (DA)	Implementing acts (IA)
health requirements for aquaculture animals and products derived therefrom, their movement within the EU and import into the EU from third countries;	surveillance, eradication, establishment of freedom from disease and control measures for certain diseases in aquaculture animals
prevention and control of certain diseases in aquaculture animals	registration and approval of establishments keeping aquaculture animals, and health requirements for the movement of aquaculture animals within the EU and import into the EU

Previous and New Lists of the Diseases of the Aquatic Animals and Their Classifications

The previous criteria on listing the diseases of aquatic animals are regulated by the Directive 2006/88/EC, whose wording distinguishes between the exotic and non-exotic diseases of aquatic animals, according to the specific criteria.

Exotic diseases (Table 3) have been defined as diseases that are not found in European aquaculture, yet the data on the distribution of a given pathogen in the European Union waters has been missing (this fact is interpreted as a lack of scientific reports or even actual reports).

Table 3

List of exotic diseases and susceptible animals in accordance with the Directive 2006/88/WE

Exotic diseases		
specification	disease	animals susceptible
Fish	Epizootic haematopoietic necrosis (EHN)	*Oncorhynchus mykiss*, *Perca fluviatilis*
	Epizootic ulcerative syndrome (EUS)	*Catla, Labeo, Mastacembelus, Mugil*, *Puntius, Trichogaster*, *Channa*
Moluscs	*Bonamia exitiosa* infection	*Ostrea angasii, Ostrea chilensis*
	Perkinsus marinus infection	*Crassostrea gigas, Crassostrea virginica*
	Microcytos mackini infection	*Crassostrea gigas, Crassostrea virginica, Ostrea edulis, Ostrea conchaphila*
Crustacea	Taura syndrome	*Penaeus setiferus*, *Penaeus stylirostris*, *Penaeus vannamei*
	Yellowhead disease	*Penaeus aztecus, Penaeus duorarum, Penaeus japonicus, Penaeus monodon, Penaeus setiferus, Penaeus stylirostris, Penaeus vannamei*

Moreover, it was emphasized that at least one of the following premises is met: a potentially significant impact of the given disease on the European Union economy if the disease is introduced in its territory, due to production losses in the aquaculture or restricted trade of aquaculture animals and the products derived from them; or a potentially negative effect on the environment if the disease is introduced in the territory of the EU and is then transmitted to wild populations of aquatic animals, particularly involving a species that should be protected by EU law or treaties.

Table 4

List of non-exotic diseases and susceptible animals in accordance with the Directive 2006/88/WE

Non-exotic diseases		
specification	disease	animals susceptible
Fish	Viral haemorrhagic septicaemia (VHS)	*Clupea*, *Coregonus*, *Esox lucius*, *Gadus aeglefinus*, *Gadus macrocephalus*, *Gadus morhua*, *Oncorhynchus*, *Oncorhynchus mykiss*, *Onos mustelus*, *Salmo trutta*, *Scophthalmus maximus*, *Sprattus sprattus*, *Thymallus thymallus*
	Infectious haematopoietic necrosis (IHN)	*Oncorhynchus keta*, *Oncorhynchus kisutch*, *Oncorhynchus masou*, *Oncorhynchus mykiss*, *Oncorhynchus nerka, Oncorhynchus rhodurus*i, *Oncorhynchus tshawytscha*, *Salmo salar*
	Koi herpes virus infection (KHV)	*Cyprinus carpio* (incl. Koi)
	Infectious salmon anemia (ISA)	*Oncorhynchus mykiss*, *Salmo salar*, *Salmo trutta*
Moluscs	*Marteilia refringens* infection	*Ostrea edulis, Ostrea angasi, Ostrea puelchana, Ostrea chilensis*, *Mytilus edulis, Mytilus galloprovincialis*
	Bonamia ostreae infection	*Ostrea angasi, Ostrea chilensis, Ostrea conchaphila, Ostrea denselammellosa*, *Ostrea edulis*, *Ostrea puelchana*
Crustacea	White Spot Syndrome	*Decapoda*

Non-exotic diseases (Table 4) are defined as diseases with the following accumulative criteria:

– absence in at least some member states or their regions,

– problems with eradication and control of the disease at the farm level or in the mollusc production area without the implementation of rigorous eradication tools and trade restrictions,

– an opportunity for the eradication of the disease at the member state level, which results from long-term experience in settling and main-

taining the zones or enclaves free from a given disease (maintenance provides the best value of money),

– there is a risk of disease transmission on previously intact areas when the aquaculture animals are marketed,

– there are reliable, simple, specific and sensitive tests for diagnosing infections in aquatic animals, and the testing method is harmonised at the European level.

Apart from the discussed data, a given disease should meet at least one of the following premises: introduction of the disease into a disease-free member state can have a significant effect on the economy due to production losses, with the annual costs associated with the disease and its eradication is more than 5% of the value of production of a susceptible aquaculture animal species in the region, or due to the restricted international trade of aquaculture animals and the products derived from them, or it has been demonstrated (which is interpreted as scientific argumentation) that introducing the disease into a disease-free member state has a negative impact in the environment and the populations of wild aquatic animals representing the species that should be protected by European law or treaties.

Importantly, the European Commission, due to lobbying by the carp producers, has decided to exclude spring viremia of carp (SVC) from the above-mentioned list (previously included in Part 2 of Annex 4 to the Directive 2006/88/EC), under the Directive 2008/53/EC of April 30, 2008.

The basis and criteria necessary to create a new list of diseases are included in Art. 5, sec. 3 of the AHL. Together with Art. 7 of this regulation, the mentioned provision represents the criteria and parameters for disease assessment and states that five accumulative obligatory criteria and at least one of the five supplemental criteria should be met (Table 5).

Viral haemorrhagic septicemia (VHS) of Salmonidae is an example of the detailed analysis of the inclusion criteria for the disease list (Barja 2004, Bale 2009, Ito 2016, Matras 2010). The etiologic virus (VHSV) is eliminated by the infected fish with urine and body fluids, so this disease is transmissible. Most species are classified in the teleost infraclass (Osteichthyes is the largest division in the Actinopterygii class, alongside the Holostei and the paraphyletic Chondrostei; it includes approx. 96% of all fish, over 2,600 species ranked in 40 orders and 448 families). Rainbow trout, turbot and olive flounder are most susceptible to infection. The disease has many negative effects on animal health: mortality varies depending on the environment and physiological characteristics, e.g. in rainbow trout, mortality can reach from 5% to 90% (regardless of the fish size).

Table 5

Method of disease evaluation based on the AHL (Art. 5), based on four primary fish diseases (positive evaluation – the disease is listed)

Disease	Mandatory, cumulative criteria (all must be met)					Additional, accessory criteria (at least one must be met)					Result
	(i)	(ii)	(iii)	(iv)	(v)	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	
KHV	+	+	+	+	+		-		-	+/-	positive
ISA	+	+	+	+	+		-		-	-	
VHS	+	+	+	+	+		-		-		
IHN	+	+	+	+	+		-		-	-	

Explanations: (i) – the scientific data show the transmissible (infectious and contagious) nature of the disease; (ii) – in the EU, there are animal species that are susceptible to this disease or are vectors or a reservoir of the disease; (iii) – the disease has negative effects on animal health or presents a risk to public health (is zoonotic); (iv) – there are diagnostic tools available for a given disease; (v) – the risk-reducing measures and allowing a reduction of the surveillance scope of a given disease in appropriate cases are effective and proportional to the risk that the disease creates in the EU; (*a*) – the disease has, or may have, substantial negative effects on animal health within the EU or represents (or may represent) a substantial risk to public health (is zoonotic); (*b*) – the disease agent has become resistant to treatment and presents a major risk to public or animal health in the EU; (*c*) – the disease leads (or might lead) to significant negative economic effects in agricultural production or aquaculture in the EU; (*d*) – the disease may cause a crisis scenario, or the disease agent can be used for bioterrorism; (*e*) – the disease has (or might have) a substantial negative effect on the environment, including biodiversity, in the EU; KHV – koi herpes virus; ISA – infectious salmon anaemia; VHS – viral haemorrhagic septicaemia; IHN – infectious haematopoietic necrosis

The diagnostic tools are based on the multiplication of cell culture and a specific and sensitive RT-PCR test as well as on the detection of the antigen (while serology is not routinely used). In the EU, there are tools for securing biological safety and reducing the risk, such as mounting nets above the fish ponds and fencing the fish farms; preventing water leaks from the trucks used to transport fish to the farms; preventing feeding the livestock fish with fresh fish and the non-transfer of live fish from sea water into fresh water. No commercial vaccine is yet available. The wide distribution of VHSV among the wild fish species in Northern Europe raises some concerns about the health status of the coastal zone and the risk of transmitting VHS into the inland waters due to upstream migrations of the diadromous fish. The disease is not zoonotic and cannot be used as a bioterrorism tool; however, it brings about significant economic consequences in the aquaculture industry, specifically in rainbow trout, olive flounder and turbot production in the 5–15°C range. Furthermore, it may have a major negative impact on the environment; particularly if genotype 4 is introduced to Europe, massive mortality in wild fish is expected (both fresh and sea water), as in the US and Canada. The disease out-

breaks caused by some VHS genotypes may have a significant (negative) effect on biodiversity.

Annex 2 of the Regulation 2016/429 (EU) has been amended with the delegated regulation of the European Commission 2018/1629 (UE), and these changes will enter into force from April 21, 2021. The amendment includes the following diseases: epizootic haematopoietic necrosis (EHN), viral haemorrhagic septicaemia (VHS), infectious hematopoietic necrosis (IHN), infectious salmon anaemia (ISA) with a deletion in the polymorphic HPR region, Koi herpesvirus, microcytosis (*Mikrocytos mackini*), perkinsosis (*Perkinsus marinus*), bonamiosis (*B. ostreae*, *B. exitiosa*), marteiliosis (*Marteilia refringens*), Taura syndrome, Yellowhead disease and *White Spot Syndrome*. Epizootic ulcerative syndrome (EUS) has been removed from the list included in Annex 2 (EFSA 2011).

The classification of diseases is the mainstay for diversified implementation and diverse effects of the legislation on the prevention and eradication of diseases, depending on the disease category featured on the list and the respectively susceptible species by Regulation 2018/1882 (EU). The regulations on prevention and eradication of the listed diseases should apply only to the species and species groups that are susceptible to these diseases or are vectors (they can transmit these diseases). The listed diseases should be addressed with different measures that are determined in the discussed regulation. These measures encompass not only the scope of basic responsibilities, such as reporting and communicating on the occurrence or suspected occurrence of the listed diseases, but also a wide scope of vigilant surveillance of the specific diseases and their eradication. They also refer to transporting the animals and animal-derived products in the EU and marketing these commodities in the EU member states from third countries.

The AHL enforces the need for authorization of the aquaculture sites (that farm the aquaculture animals for transferring them live or as the animal-derived aquaculture products from the site) by the District Veterinary Officer (Art. 176). To this end, there is also a need to meet the specific requirements referring to husbandry, such as biosecurity, by the fish farms (Art. 177–184).

The Art. 8, secs. 2 and 3, And art. 9, secs. 1 and 2, and Annex 4 to the AHL specify the inclusion criteria for the species or species groups and the methods for applying the regulations on disease prevention and eradication, as well as the rules for implementing individual provisions, depending on the disease category.

The listed disease categories have been legislated in art. 9, section 1, items a-e of the AHL, and subsequently, they have been clarified in the Regulation 2018/1882 (EU) as follows:

– Category *A* includes diseases that do not typically occur in the EU member states, and the immediate eradication measures to eliminate the disease outbreak must be implemented when the disease is detected,

– Category *B* includes diseases that must be eradicated in all EU member states (art. 9, section 1, item b of the AHL),

– Category *C* includes diseases that are important only in some of the member states, and their further transmission into the other parts of the EU must be prevented (areas that are officially free from the disease; areas in which the eradication plans have been implemented),

– Category *D* includes diseases for which the transmission must be prevented due to their occurrence in, or transfer between, the EU member states,

– Category *E* includes the diseases that must be surveyed.

It is possible to classify a given disease in one or multiple categories, e.g. by the above-mentioned norms: Koi herpes virus is considered a Category *E* disease; epizootic haematopoietic necrosis, microcytosis, dermo disease (perkinsosis), Yellowhead disease and Taura syndrome are categorized in the *A*, *D* and *E* groups; and viral haemorrhagic septicaemia, viral haemorrhagic septicaemia of the Salmonidae, infectious salmon anaemia, WWS, marteiliosis and bonamiosis are classified in *C*, *D* and *E* groups.

The regulations on the prevention and eradication of diseases are applied to the listed diseases, while the actual implementation of these provisions depends on the disease category and the animal species group in which there is a substantial risk of disease transmission and transmission of animals spreading the disease (vectors).

Conclusions

In European law, there are noticeable tendencies that, to put it simply, can be called *codifying*. The meticulous analysis of the measures, methods, specific objectives and scopes of the individual procedures undertaken by the EU bodies leads to the conclusion that by nature, they are merely compilatory and aimed at compiling and partly systematizing the existing and fragmented legislation that has been created throughout the whole European Union development into its current legal and system model. Furthermore, a major part of these procedures do not cover or address the wholeness of the discussed subject; even if the title or preamble of the given legislative act represents it as being comprehensive legislation on a certain subject, an analysis of the individual provisions of the legislative act does not indicate a holistic approach or the exhaustive codification of a certain part of the law.

As a matter of fact, the EU authorities strive to unify the regulations in the member states, particularly in such an important area – from the veterinary perspective as well as the public health – as the health of fish and mollusc. However, the measures undertaken by the EU bodies happen to be inadequate to the assumed targets. There is also a lack (or excessive procrastination) of legislative work, which is required by law, on the secondary legislation and implementing legislation, both on the European and national levels.

Even though the discussed laws on animal health warrant editorial revision and further complementation referring to registration, authorization and identification of animal movement, in their existing form, they still encompass dozens of previous normative acts and represent the legal norms that facilitate using the veterinary laws, including those referring to the diseases of animals.

The advantage of the investigated legislation is an approach that addresses the broad relationship between public health and the environment using the conceptualization of biodiversity and varied surveillance and control. The inclusion of fish and mollusc diseases in the legislation associated with the regulations on the health of the other animal is evidence of their common and progressive integration. This has been reflected in the legal provisions on management and control in animal health and production, both for the terrestrial and aquatic species.

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EXTRACELLULAR ANTIBACTERIAL DEFENSE MECHANISMS OF NEUTROPHIL GRANULOCYTES AND THEIR ROLE IN PATHOGENESIS OF PYOMETRA (CASES) IN CATS

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Key words: cats, pyometra, local immunity, phagocytes, neutrophil extracellular traps.

Abstract

Pyometra in cats is the most common reproductive pathology with septic inflammation in uterus, accompanied by a cascade of immunological responses and changes in local homeostasis. The aim of the research is to study the extracellular protective mechanisms of neutrophil granulocytes in the course of immunological response during the development of pyometra in cats. The observed acute inflammatory reaction in the infected organism was accompanied by the active migration of phagocytes to the pathological to the site of inflammation. The pathology was demonstrated by the active growth of cytochemical reactivity of the Oxygen-dependent antimicrobial potential of the neutrophils in genital mucosa and the initiation of extracellular protective trap formation mechanism. Cytological markers of phagocytic cells detected in the pathogenesis of the pyometra should be taken into account during the diagnosis of the pyometra, prognosis of the course of this reproductive pathology and analysis of the adequacy of therapy.

Introduction

Pyometra is common reproductive pathology in cats and is characterized by cystic endometrial hyperplasia and septic inflammation which develops secondary to hormone-dependent alteration of endometrium

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(Dmirel and Acar 2012, Hollinshead and Krekeler 2016, Zhelavskyi and Shunin 2017). Pathogenesis of the pyometra is complex and can be described by the development of dysfunctions in all organs and systems (Chen et al. 2012, Graham and Taylor 2012, Hagman 2018). Despite this, immune defense mechanisms are of crucial importance in pathogenesis of this condition (Maciel et al. 2014, Grayson and Kaplan 2015, Jursza et al. 2015).

Neutrophils are a population of immunocompetent cells that have a number of membrane receptors on their surface and are able to respond to changes in homeostasis (Khan et al. 2011, Kaplan and Radic 2013, Jursza-Piotrowska and Siemieniuch 2016). Neutrophil granulocytes are the first-responders during inflammation (Caudrillier et al. 2012, Chen et al. 2014), which first migrate into the pathological process area and realize their phagocytic function (Wira et al. 2005, Gould et al. 2014).

In infected tissues, neutrophilic granulocytes destroy microorganisms by involving cellular and extracellular mechanisms of antimicrobial defense. The study of the structure, physiology of neutrophilic granulocytes, the their biochemical composition, and the mechanisms of interaction is very relevant (Akong-Moore et al. 2012, Gray et al. 2013, Chen et al. 2014). Phagocytes is has by the active role that they play in maintaining the homeostasis of the body (Parker al. 2012, Kaplan and Radic 2013). The main function of neutrophilic granulocytes is phagocytosis. The objects of phagocytosis are usually biological agents having a corpuscular structure (bacterial and fungal pathogens, protozoan cells, their own damaged cells and their decay products). Neutrophils absorb and digest captured microorganisms using Oxygen-dependent and Oxygen-independent mechanisms, which leads to their elimination (Wira et al. 2005, Zhelavskyi and Shunin 2017, Vilhena et al. 2018).

The neutrophil cytoplasm contains three main types of granules – primary (azurophilic), secondary (specific) and tertiary. Primary azurophilic granules include myeloperoxidase (MPO), which is necessary for the enzymatic conversion of H_2O_2 to HOCl. They also contain harvested neutrophil elastase and defensin proteins. Defensins are embedded in the microbial membrane with a violation of its integrity. They can also destroy the DNA of bacteria (Caudrillier et al. 2012, Metzler et al. 2014, Jursza et al. 2015). Secondary granules contain lysozyme, lactoferrin, gelatinase and metalloproteases. Their membranes also contain up to 95% cytochrome B558, a component of the NADPH oxidase enzyme (nicotinamide adenine dinucleotide phosphate oxidase). The main enzyme of tertiary granules is gelatinase (Khan et al. 2011, Kahlenberg et al. 2013, Kenny et al. 2017).

Neutrophils have the ability to respond even to minor environmental changes using an extensive array of membrane receptors from different families (FUTOSI et al. 2013, CHU et al. 2016). On their membranes are presented, for example, Toll-like receptors (TLR), Fc receptors for immunoglobulins of various classes, primarily IgG, receptors for complement components (C3b and others), which ensures efficient opsonization of phagocytosis objects (JAILLON et al. 2016, NOWAK et al. 2019). In infected tissues, neutrophilic granulocytes collide with microorganisms and are activated, absorbing the pathogen in vacuoles (phagosomes). Further, neutrophil granules merge with the phagosome, forming phagolysosomes into which antimicrobial peptides and enzymes fall. Moreover, in the phagolysosome, microorganisms are exposed to high concentrations of reactive oxygen species (ROS) (KAMBAS et al. 2012, DIANA et al. 2013, PEREZ-DE-PUIG et al. 2015). For a long time, neutrophils were considered only as nonspecific effector cells of innate immunity, realizing all of the above functions. After completing his biological program, the neutrophil dies. Apoptosis after phagocytosis or possible necrosis under the influence of the pathogenicity factor of pathogens was considered the most probable outcome of differentiated neutrophils (CAUDRILLIER et al. 2012, KIDD et al. 2013, HARBORT et al. 2015).

In 2003 VOLKER BRINKMANN (Max Planck Institute For Infection Biology, Germany) a new mechanism for the antimicrobial action of neutrophils has been described. It turned out that neutrophilic granulocytes after activation eject network-like structures into the extracellular space (KNIGHT and KAPLAN 2012). Space, which include DNA, histones, as well as various proteins and granule enzymes, such as elastase and myeloperoxidase. These structures were called "neutrophilic extracellular traps" (Neutrophil Extracellular Traps, NETs) (HARBORT et al. 2015, AMULIC et al. 2017). Initially, the purpose of this phenomenon was unclear. Nevertheless, it was immediately suggested that network-like structures isolate and destroy gram-positive and gram-negative bacteria, fungal pathogens. It should be noted that processes resembling the formation of NETs also mentioned in earlier sources.

The integrity of the outer membrane of granulocytes activated in this way does not affect the destruction of the inner membranes that allow mixing of the intracellular components of the neutrophil. This process directly depends on the formation of ROS. Most likely, this is one of the first descriptions of the formation of NETs (DE MEYER et al. 2012, KAHLENBERG et al. 2013). The formation of extracellular traps (or NETosis, "netosis") is another variant of the fate of a neutrophilic white blood cell (CHOWDHURY et al. 2014, GRAYSON and KAPLAN 2016).

It is known that the intensity of the inflammatory reaction depends largely on the cascade of immunological *responses* in which the cellular mechanisms of protection are involved (KHAN et al. 2011, PARKER et al. 2012, ZHELAVSKYI and SHUNIN 2017). In view of this, neutrophil granulocytes play an important role in maintaining homeostasis. At the same time, the cellular factors of local immunity of reproductive organs of cats are still not sufficiently studied, which makes for the necessity of a detailed research of the mechanisms of antimicrobial protection both at normal state and with the pathogenesis of the pyometra. Consequently, the functional capacity of phagocytes is an important indicator value. Its exploration is promising for the detection of informative cytological markers of inflammation and will be useful for improving the methods of cat pyometra diagnosis.

The aim of the research is to study the extracellular protective mechanisms of neutrophil granulocytes in realization of local immunity during the development of pyometra in cats.

Materials and Methods

Animals' criteria. Clinical and experimental studies were a performed in the veterinary clinic and in the Specialized Laboratory of Immunology of Reproduction Animals (State Agrarian and Engineering University in Podilya, Kamianets-Podilskyi, Ukraine). For the experimental part of the work, control groups (healthy, n = 17) and experimental (with an open form of the pyometra n = 17) of cats were formed.

The diagnosis of the pyometra was based on interview (history), clinical symptoms, laboratory (cytological, microbiological, haematological, immunological) and ultrasound examination (Toshiba Core Vision Pro, Japan, linear transducer 8-MHz).

Blood collection and analyses. Blood samples for haematological analysis were tested in the Specialized Laboratory of Immunology of Reproduction Animals, Department of Veterinary Medicine, State Agrarian and Engineering University in Podilya, Ukraine. The above mentioned analyses were performed using routine methods, parameters (RT-7600 VET) included haemoglobin (Hb), haematocrit, red blood cell count (RBC), white blood cell count (WBC), differential count of WBC including total count (BN) and percentage band neutrophils (PBN).

Determination of antimicrobial potential of neutrophils in reaction with nitro blue tetrazolium (NBT-test). Using a special brush for cytology pre-wetted with 15 M phosphate buffer NeoGalin18 (15 M NaH_2PO_4 + $2H_2O$ (11.8 g) + KH_2PO_4 (68.0 g) + C_6H2O_6 (10.0 g), pH 7.2) samples from

dorsal parts of the vagina, cervix and the exudate were obtained from the uterus, which were applied to the microscope slide. 0.05 ml of 0.15% solution of nitro blue tetrazolium (produced by Renal®, UK; phosphate buffer (pH 7.2) were added to the cell mixture. Subsequently, the microslides were incubated for 30 minutes in a wet chamber of the thermostat (37°C). After incubation, smears stained with methanol and stained with 0.1% phosphate buffered neutral red (pH 7.2) were prepared (ZHELAVSKYI 2017).

Assessment and accounting of metabolic reactions of phagocytes (determination of the percentage of reactive neutrophils) were carried out microscopically (2500 x magnification). Reactive (NBT reactive) neutrophil granulocytes were visualized by the presence of dark-brown inclusions in the cytoplasm in the cytoplasm in the form of fine diffuse grains. The intensity of the "respiratory burst" (the percentage of active microphages that showed reactivity) was determined using IV stages. A zero (0) degree was characterized by a complete absence of granulomas of formazan in phagocytic cytoplasm cells. Such microphages were classified as intact. The first (I) degree was manifested by the formation of single granules in phagocytic cells. The second (II) – in the presence of inclusions occupying almost 1/3 of the cytoplasm. To the third (III) degree belonged cells in which cytoplasmic inclusions occupied 2/3 of the cell area. The highest IV level of metabolic reactivity of phagocytes was expressed by the formation of intense, well-expressed granules of diformasan, which were visualized throughout the cytoplasm, including the nucleus of microphage cells (KENNY et al. 2017).

While determining the degree of reactivity and interpretation of cytochemical indicators, following indices were calculated: the cytological index (CLI [%]; index of activation of neutrophils (IAN in standard units of measurement (c.u.); the index of migration activity of neutrophils (IMN in standard units of measurement (c.u.) and the ratio of phagocytes to epithelial cells (Fag/Epithel) (ZHELAVSKYI 2017).

Determining the ability of neutrophils to form NETs (Neutrophil extracellular traps). The diagnostic material was obtained from the vaginal mucosa taken with a cytologic brush pre-moistened with 15 M phosphate buffer NeoGalin18 (pH 7.2). Then a smear was prepared. After drying at room temperature (20°C), the microslide was fixed with methanol and stained with 1% phosphate buffered saline solution (pH 7.2) at exposure for 2–3 min. After that, the *microslide* was rinsed with phosphate buffer and stained with a dye-fixative eosin methylene blue for May-Grünwald. Estimation of neutrophils from NETs [%] was carried out microscopically – 2500x magnification (ZHELAVSKI 2017).

Results

The cat's pyometra is usually observed at the age from 3 to 10 years. During the entire observation period (2014–2019), the disease was registered in 382 cats. The disease was manifested in animals from 3 years. The open form of the pyometra was observed 14.1±0.72%. The largest risk group was the 5 years old animals (16.7±0.47 %) and the incidence of disease continued to decrease gradually.

Signs of the illness were diagnosed in the luteal phase. Breed predisposition detailed study, the animal's predisposition to the development of the pyometra was established (Table 1). Most often reproductive pathology was manifested in *Persian breed* (24.2±0.62%), *Turkish Angora* (20.7±0.57%) and Siamese (19.3±0.52%).

Table 1

Breed predisposition of cats to the pyometra (Mean±SD)

Breed	Frequency of pyometra [%]
Turkish Angora	20.7±0.57
European group	7.8±0.27
Brittan	15.8±0.37
Persian	24.2±0.62
Siamese	19.3±0.52
Domestic cat	12.2±0.42

It was found that in open form of the pyometra the main symptoms of the disease in animals include lethary/depresssion, fever, tachycardia, dysuria, abdominal distension. The most clinical symptoms were haemopurulent vulvar discharge, hyporexia/anorexia, vomiting and weight loss. Ultrasonography has revealed the presence of fluid within the lumen of the uterus. The uterine wall often appeared thickened with irregular edges and small hypoechoic areas consistent with cystic changes to the endometrial glands.

In a haematological examination, an increase in the number of leukocytes ($33.01\pm1.27 \cdot 10^9$/L, $P < 0.01$) and signs of severe neutrophilia (75.88±0.99%, $P < 0.01$) were observed. Moreover, red cells amount ($5.17\pm0.25 \cdot 10^{12}$/L, $P < 0.05$), haemoglobin and haematocrit lever decreased as well (Table 2). Acute inflammatory reaction was accompanied by active migration of phagocytes into the area of the pathological process (IMN, Figure 1). In microslides taken from the vaginal mucosa, an increase in the number of neutrophilic granulocytes was observed (26.23±1.03%, $P < 0.01$, Table 3). The inflammatory response was accompanied by a cell imbalance with an increase in number of phagocytes (Phag/Epithel 1.14±0.04, $P < 0.05$) and IAN (0.34±0.01, $P < 0.01$, Figure 2).

Table 2

Hematological indices of cats with a pyometra (Mean ± SD)

Variables	Healthy feline (n = 17)	Hospitalized feline (n = 17)
WBC [$\cdot 10^9$/L]	17.05±0.74	33.01±1.27**
RBC [$\cdot 10^{12}$/L]	7.21±0.42	5.17±0.25*
Hemoglobin [mmol/L]	11.72±0.53	7.21±0.27**
Hematocrit [L/L]	23.15±0.52	38.17±0.67*
Neutrophils [%]	46.01±0.86	75.88±0.99**

n – number; *P < 0.05; **P < 0.01; WBC – white blood cells; RBC – red blood cells

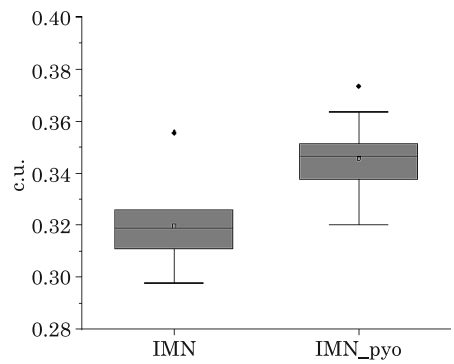

Fig. 1. Migratory activity of neutrophil granulocytes (Mean ±SD): IMN – index of migratory activity of neutrophils; IMN_pyo – index of migratory activity of neutrophils, patients with cats' pyometra; c.u. – conditional units of measurement

Table 3

Cytological indices of the mucous membrane of the vagina of cats with a pyometra (Mean ± SD)

Variables	Healthy cats (n = 17)	Hospitalized cats (n = 17)
Neutrophils mucosa [%]	14.70±0.68	26.23±1.03**
Phagocyte/Epithelial cell	0.17±0.09	1.14±0.04*

n – number; *P < 0.05; **P < 0.01

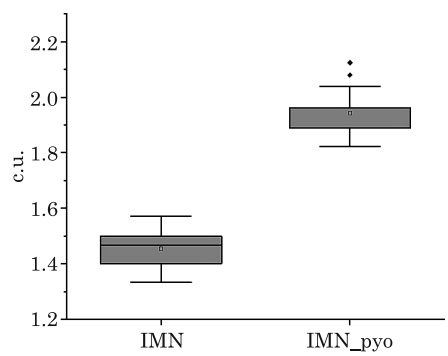

Fig. 2. Activation of phagocytic activity during pyometra (Mean ± SD): IAN – neutrophil activation index; IAN_pyo – index of activation of neutrophils, patients with cats' pyometra; c.u. – conditional units of measurement

The pathological process was manifested by the growth of cytochemical reactivity neutrophils (NBT 50.88±0.85%, $P < 0.01$) with the highest activity of III (18.41±0.50%, $P < 0.01$) and IV (14.29±0.77%, $P < 0.01$). In this case, the CLI increased significantly to 1.43±0.02, $P < 0.01$, Table 4).

Table 4

Indicators of antimicrobial activity of Oxygen-dependent mechanisms of protection of healthy cats and hospitalized whith pyometra (Mean ± SD)

Variables	NBT-test [%]	Cytochemical reactivity [%]					
		0 stage	I stage	II stage	III stage	IV stage	CLI
Healthy cats (n = 17)	21.35±0.86	78.64±0.86	10.41±0.50	7.64±0.49	2.29±0.46	1.01±0.35	0.36±0.02
Hospitalized cats (n = 17)	50.88±0.85**	77.94±0.76	2.64±0.49**	18.41±0.50**	15.52±0.51**	14.29±0.77**	1.43±0.02**

n – number; NBT-test – cytochemical reactivity of neutrophils in reaction with nitro blue tetrazolium; 0–IV stage – step intensity of cytochemical reactivity of neutrophils; CLI – cytological index; **$P < 0.01$

The number of neutrophilic granulocytes NETs (61.94±0.89%, $P < 0.01$; Table 5) increased on the mucous membranes.

Table 5

NETs activity of neutrophils of the mucous membrane of cats' genitals with pyometra (Mean±SD)

Variables	Healthy cats (n = 17)	Hospitalized cats (n = 17)
NETs [%]	27.05±0.82	61.94±0.89**

n – number, NETs – neutrophil extracellular traps; **$P < 0.01$

Endometrial inflammation was also manifested by an exudative reaction with active involvement of phagocytic cells in the pathogenic pathology area (NETs, Figure 3 and Figure 4).

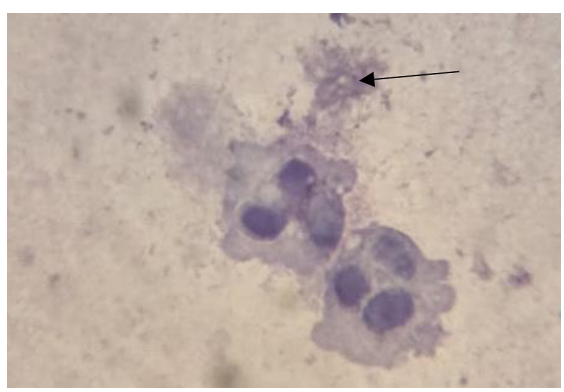

Fig. 3. Formation of protective traps NETs (black arrow) by microphages of the uterine mucosa (2500x magnification with Malachite green and May-Grünwald staining)

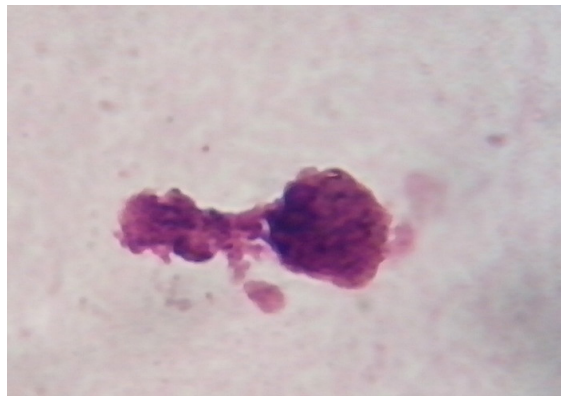

Fig. 4. Neutrophil granulocyte with NETs (2500x magnification with May-Grünwald staining)

Discussion

Scientists from many countries around the world are focused on the study of local factors in the protection of reproductive organs of animals (WIRA et al. 2005, MACIEL et al. 2014, JURSZA et al. 2014). More attention is paid to cellular mechanisms of protection as one of the components of immune homeostasis (RISSO et al. 2014, LI and TABLIN 2018). There are isolated reports on the state of phagocytic protection of the mucous membranes of the reproductive organs of animals in different periods of the oestral cycle and in the development of the pyometra (REBORDÃO et al. 2017, PAPAYANNOPOULOS 2018).

Neutrophil products secreted into the trap space have selective bactericidal properties. They inhibit the growth of pathogenic and conditionally pathogenic microorganisms, but have little effect on non-pathogenic microorganisms, in particular lactic or bifidobacteria (JAILLON et al. 2016). This may be due to differences in the mechanisms of neutrophil stimulation by pathogenic and non-pathogenic pathogens. It was previously found that intracellular neutrophil killer systems are more activated by *E. coli* and *S. aureus* than by *Lactobacterium spp.* and *Bifidobacterium spp*. (MANTOVANI et al. 2011, JAILLON et al. 2016). In addition, cells secrete a different set of antimicrobial mediators upon neutrophil activation through separate groups of receptor molecules, such as image-recognition receptors. Bacterial opsonization (eg, interaction of specific IgG with the envelope *S. aureus*) also stimulates the production of NETs by acting through Fc receptors of leukocytes (KRAMER et al. 2017). Obviously, along with microbial stimulation, trap formation is also regulated by numerous external factors and signaling. Pro-inflammatory agents such as interleukin-8,

lipopolysaccharide (LPS) or phorbolmyristate acetate are provoked by the formation of traps. In turn, the NADPH oxidase inhibitor diphenylene iodone prevents the formation of NETs. Separately, IL-8 and LPS release traps less efficiently than bacteria. Optimal leukocyte NETs formation requires activation through several receptors (FUTOSI et al. 2013, JAILLON et al. 2016). On the other hand, it turned out that neutrophils of whole blood and its leukocyte suspension did not normally form spontaneous NETs, despite the periodic increase in the concentration of activators in serum (KHAN et al. 2011, DE MEYER et al. 2012, HAZELDINE et al. 2014).

Moreover, the process of death of granulocyte significantly differs from apoptosis and necrosis, which were studied previously (GRAHAM and TAYLOR 2012). Studies have shown that network formation is a controlled process, and not an accidental release of granules and nuclear contents of a cell, as during necrosis or apoptosis. It has been established that networks can form as an alternative to phagocytosis. Compared to apoptosis and necrosis, the most important morphological differences in netosis are decay of the nuclear membrane and mixing of nuclear and cytoplasmic material, loss of the inner membrane and disappearance of cytoplasmic organelles. Neutrophil apoptosis is a strictly regulated response that seeks to prevent cell contents from entering the intercellular space. NETosis, in contrast, is directed to the controlled release of intracellular granulocyte components. This process is also subject to strict regulation. Unlike apoptosis, it is stimulated by ROS, but does not depend on caspases. In this case, DNA fragmentation does not occur, but nuclear membrane destruction is observed (KNIGHT et al. 2012, BRODZKI et al. 2015, HARBORT et al. 2015, LI et al. 2018).

During neutrophil activation, a cellular signaling system is induced, including phosphatidyl-inositol-3-kinase and serine-threonine kinase (STK), which is responsible for protein synthesis, microtubule function, and neutrophil autophagy. This system takes part in the disintegration and rupture of the cell membrane during "netosis" (CHOWDHURY et al. 2014, YAN et al. 2015). After activation of the cytoskeleton, the cell contracts until the outer membrane ruptures. A highly active mixture, once in the extracellular space, forms a peculiar three-dimensional network, a "trap", into which bacteria enter. The neutrophil dies. This oxygen-dependent cell death was called the term "NETosis" (FUCHS et al. 2012, MCINTURFF et al. 2012, GOULD et al. 2014). This suggests that in the bloodstream of healthy animals, the formation of NETs should not occur. Many authors have shown that the formation of NETs in the bloodstream mechanically disrupts blood circulation in the tissues and organs (REBORDÃO et al. 2017, PAPAYANNOPOULOS 2018). Blood inhibitory factors have

been found to have a humoral nature. Autologous serum and blood plasma inhibit extracellular DNA release by neutrophils isolated from peripheral blood. Thus, in the systemic blood flow, in the absence of inflammation, the formation of NETs is suppressed (MACIEL et al. 2014, JEFFERY et al. 2016). Neutrophils of patients with chronic granulomatous disease are known to be unable to generate ROS due to a deficiency of the NADPH oxidase enzyme. In turn, the neutrophils of these patients were unable to form NETs. However, at least in part, the glucose oxidase enzyme compensated for the functional failure of NADPH oxidase to produce hydrogen peroxide (YAN et al. 2015). However, under the influence of glucose oxidase, the formation of NETs significantly increased. In connection with all the above data, it can be concluded that, on the one hand, NETs function as an effective antimicrobial barrier, on the other hand – their excess leads to the development of inflammatory processes and to hemodynamic disorders in case of deficiency of counteracting regulatory mechanisms. Neutrophils are, above all, tissue cells involved in inflammatory and antimicrobial reactions. They also function actively in the mucous membrane (CAUDRILLIER et al. 2012, GOULD et al. 2014). It is obvious that disorders of mucosal immunity contribute to the recurrent course and chronicity of local inflammatory processes (GRAY et al. 2013).

According to some authors, the formation of NETs is a mechanism of protection that acts in the tissues and on the surface of the mucous membranes, and is especially important in the mucosal anti-infective protection (JEFFERY et al. 2016). Neutrophils leaving the tissues and leaving the mucous membranes can participate in the antimicrobial protection and in the regulation of the microbiota of the respective biotopes, secreting biocidal products (JURSZA et al. 2015, ZHELAVSKYI 2017). In addition, the aggressive factors of neutrophil granules in the trap formed are linked by DNA strands. In the case of colonization of tissues or mucous membranes by representatives of normal microflora, trap components are not capable of causing the development of inflammatory responses (MANTOVANI et al. 2011, ZHELAVSKYI and SHUNIN 2017, Li et al. 2018).

In the presented study the state of phagocytic protection of mucous membranes of genitalia of cats during development of a pyometra is considered. In our studies, it has been found that in the pathogenesis of the pyometra there is a cascade of immune reactions. Neutrophilic granulocytes are actively migrating from the peripheral blood to the zone of the pathological process. The launch of phagocytic reaction takes place. Activated neutrophils carry out an active attack of microorganisms and involve extracellular mechanisms of protection (PARKER et al. 2012, HAZELDINE et al. 2014, JURSZA et al. 2015).

In the extracellular space, phagocytes excrete a number of antimicrobials, including reactive oxygen species (ROS) (De Meyer et al. 2012, Zhelavskyi and Shunin 2017). Our study found that the total number of activated neutrophils of mucosal surfaces of reproductive organs can excite generation of ROS. At the same time, the inflammatory reaction was accompanied by activation of the formation of NETs. This, in our opinion, is induced by pathogenic strains of microorganisms that have penetrated the zone of the pathological process.

The process of creating NETs begins with the activation of neutrophil. The launch of the membrane-binding multimolecular enzyme complex NADPH oxidase takes place. "Respiratory burst" is being activated. Formulated by ROS (Hazeldine et al. 2014, Luo et al. 2014, Jeffery et al. 2016) that induce activation of enzyme systems of phagocyte (elastase and Protein arginine deiminase 4 (PAD-4) (Wira et al. 2005, Papayannopoulos 2018). There is a conversion of arginine and methyl arginine to cerulin in the histone proteins of the nucleus. The consequence is the decomposition of chromatine and the release of DNA (Kaplan and Radic 2012, Luo et al. 2014, Martinod and Wagner 2014).

In the process of activating neutrophils, a cellular signaling system, including phosphatidylinositol-3-kinase and sertronicin kinase (STK), which is responsible for protein synthesis, microtubule function and neutrophil autophagy, is induced. This system participates in the disintegration and rupture of the cell membrane during the "NETosis" (Parker et al. 2012, Mcinturff et al. 2012, Papayannopoulos 2018).

After the activation of the cytoskeleton, the formation of a volumetric grid, a "trap", occurs in which bacteria enter. Neutrophil dies at the same time. In the course of the formation of NETs, in conjunction with decondensated chromatin (DNA and histones), proteases and antimicrobial peptides, that are contained in neutrophil granules, are released. These indicators are important markers of inflammation (Jursza et al. 2015, Jeffery et al. 2016, Zhelavskyi 2018). Recent studies have proven the importance of cellular protective factors for local immunity of the animal's genital organs. studies have shown that neutrophil granulocytes are the primary messengers of the inflammatory process. Microphages actively migrate from the peripheral bloodstream. Neutrophil granulocytes show active protection through the realization of extracellular protection factors. Changes in cytochemical markers can be taken into account for the diagnosis of pyometra (subclinical manifestations, closed pathology). And as well can be taken into at the prognosis of this reproductive pathology (Zhelavskyi 2019). On the other hand, it can be used to analyze immunological shifts and develop adequate therapy.

Conclusion

Cat's pyometra is a widely spread reproductive disease that occurs due to the changes in endocrine regulation and immune homeostasis. Cascade of disturbances of mechanisms in local immune protection of the uterus occurs in the pathogenesis of the disease. The antimicrobial potential of neutrophils was realease of extracellular defense by activating extracellular defense mechanisms with an active excretion into the extracellular space of the active forms of oxygen and the release of extracellular protective traps. Cytological markers of phagocytic cells (NBT-test, NETs) proved to play or role in pathogenesis of the pyometra should be taken into account during the diagnosis of the pyometra, prognosis of the course of this reproductive pathology and analysis of the adequacy of therapy.

Ethical Approval

This study was approved according to the Law of Ukraine "On the Protection of Animals from Cruel Treatment" (No. 3447-IV of February 21, 2006) and according to the requirements of the European Convention for the Protection of Pet Animals (ETS No. 125, Strasbourg, 13/11/1987). All experiments were carried out with the Ethical Permit at the State Agrarian and Engineering University in Podilya, Ukraine and an informed consent was obtained from the owner prior to the inclusion of the cats in the study. All animal manipulations were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and scientific purposes (Strasbourg, 18 March 1986).

Conflict of interest. The author declare that there is no conflict of interest.

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