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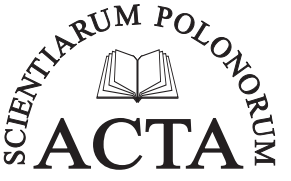



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# ENRICHMENT OF HEN EGGS WITH OMEGA-3 POLYUNSATURATED FATTY ACIDS – PHYSIOLOGICAL AND NUTRITIONAL ASPECTS*

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**Abstract.** The biological role of omega-3 acids, α –linolenic (ALA, C18:3 n-3), eicosapentaenoic (EPA, C20:5 n-3), docosapentaenoic (DPA, C22:5 n-3) and docosahexaenoic (DHA, C22:6 n-3) in human organism was described. The sources (sea organisms – fish and algae as well as oily plants, mainly linseed) and their possible applications in feeding layer poultry in order to enrich hen yolks with the acids was described. Moreover, the problem of the so called fish-taste and smell as well as the methods of limiting such negative sensory features were discussed.

**Key words:** egg, polyunsaturated fatty acid, feed

## INTRODUCTION

Customers have recently been more and more interested in food products containing polyunsaturated omega-3 fatty acids (PUFA n-3) which play a very important biological role in ensuring proper physiological functioning of human body [Newton 1966, Ruxton at el. 2004, Elmadfa and Kornsteiner 2010].

The following acids belong to the omega-3 (n-3) family: α–linolenic (ALA, C18:3), eicosapentaenoic (EPA, C20:5), docosapentaenoic (DPA, C22:5), docosahexaenoic



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(DHA, C22:6). The three last acids are the so called long-chain polyunsaturated fatty acids (LC- PUFA n-3) [Meyer et al. 2003]. They have 18 carbon atoms in a chain and more than three unsaturated bonds. Alpha–linolenic acid is a biological precursor of long-chain acids. The transformation of ALA to LC-PUFA n-3 in the organism is through elongation of the carbon chain and through the creation of additional double bonds (desaturation) [Burdge 2004]. According to literature, absorption of ALA from food in humans, calculated using $^{13}C$ method, is ca. 96%, but the conversion to EPA and DHA is very low. Some sources indicate that the conversion of ALA to DHA in humans is only 0.05–0.1% [Pawlosky et al. 2001, Burdge 2006], whereas the conversion of ALA to EPA and DHA is at the total level of 0.4% [Hussein et al. 2005]. Other authors [Schreiner et al. 2004] indicate the level of conversion of both acids at a higher level of 5–10%. Such low level of biotransformation results from, among others, strong competition for enzymes between omega-3 fatty acids (ALA) with omega-6 fatty acids, mainly linoleic acid (LA C18:2) and arachidonic acid (AA C20:4) prevailing in diet. The enzyme they mainly compete for is delta-6-desaturase [Simopoulos 2002].

Information about the deficiency of omega-3 acids in human diet and its health results is frequent, not only in medical literature. This situation is caused by insufficient daily consumption of products containing these acids, including seafood (mainly fish) as well as some plant oils. That is why enrichment of products of animal origin, such as hen eggs, milk or meat, is of special importance [Palmquist and Griinari 2006, Woods and Fearon 2009]. Eggs are a perfect carrier of numerous important components, e.g. amino-acids, vitamins, omega-3 acids and other nutraceutical substances [Shapira 2010, Sosnowska et al. 2011]. Changing the content of fatty acids in egg yolk through supplementation of feed with components rich in omega-3 acids may be an efficient method for the production of eggs enriched with LC-PUFA – fatty acids necessary for proper human metabolism [Trziszka 2000].

## BIOLOGICAL ROLE OF OMEGA-3 ACIDS

The role and biological importance of polyunsaturated fatty acids, mainly omega-3, are subject of numerous studies and publications. It is known that they are the starting material in the synthesis of tissue hormones: prostaglandin, prostacyclin, thromboxane and leukotriens (eicosanoids). These hormones influence the level of adenosine monophoshate (AMP) in the cells, inhibit aggregation of blood platelets, control the contractility of the smooth muscles of the vascular walls and heart, regulate blood pressure, prevent intravascular clotting and inflammations. The consumption of omega-3 acids reduces the risk of heart diseases, inhibits the growth of prostate and breast cancer, helps the functioning of the immune system and is necessary to ensure the proper functioning of brain (maintenance of learning memory performance) and retina [Lewis et al. 2000, Burdge 2006, Su 2010].

It should also be mentioned that there is a growing number of reports about anticancer and prophylactic role of oleic acid (OLA C18:1, n-9), found mainly in olive oil, rapeseed and in hen yolk, in treating heart diseases [Waterman and Lockwood 2007, Shapira 2009].

Omega-6 PUFAs (mainly LA and AA) are said to have the ability to lower the level of cholesterol in blood by, among other, increasing the secretion and excretion of cholic acids (containing cholesterol) with faeces. Unfortunately, omega-3 acids induce the oxidation of low-density lipoproteins (LDL) which probably play a key role in pathophysiology of arteriosclerosis [Stocker and Keaney 2004]. There are reports that saturated acids (SFA), esp. palmitic acid and, to a lower degree, stearinic acid, bring about an increased level of cholesterol in human blood [Ruxton at al. 2004, Bałasińska et al. 2010].

In order to ensure the balance of the processes presented above, it is necessary to keep the proper n-6/n-3 ratio in the diet. This ratio in human diet should not be more than 9:1, preferably 4:1 [Simopoulos et al. 2002]. The contribution of acids in overall energy balance of a diet and their daily intake are important issues.

In many developed countries, the consumption of long-chain omega-3 acids was 0.15 g/day avg. and was below the recommended level [Kolanowski et al. 2004]. The highest consumption of EPA+DHA acids is observed in Norway and Japan, and very low in vegetarians in the USA [Elmadfa and Kornsteiner 2010]. Nutrition specialists suggest increasing the consumption of omega-3 acids. According to EANS (European Academy of Nutritional Sciences) and British nutritional recommendations, the intake should be avg. 0.2 g/day EPA + DHA/day [de Deckere et al. 1998, Ruxton et al. 2004]. The recommendations of ISSFAL (International Society for the Study of Fatty Acids and Lipids) concerning the consumption of omega-3 acids for adults are 0.65 g/day DHA + EPA (minimum 0.22 g/day of each acid). [Simopoulos et al. 1999]. According FAO/WHO so called AMDR (acceptable macronutrient distribution range) for EPA+DHA is 0.25–2.0 g/day, together with others fatty acids intake, including ALA. The maximum consumption of EPA + DHA should not exceed 3.0 g/day, because higher dose reduce cytokine production and increase lipid peroxidation [Elmadfa and Kornsteiner 2010].

According to the analysis conducted based on the data obtained from the Main Statistical Office, the average consumption of polyunsaturated fatty acids in Poland is ca. 1.5 g in case of omega-3 ALA and as little as 0.15 g EPA and DHA, thus the n-6/n-3 ratio in diet is ca. 9:1 [Kolanowski 2007].

The participation of products rich in omega-3 acids, mainly of marine origin (fish and fish products) as well as some plant oils (including linseed oil and olive oil), should be increased in human diet. However, one should be aware that, due to various reasons, it is often impossible. Thus, other methods leading to the introduction of n-3 acids to everyday food products, including eggs enriched with PUFA/LC-PUFA as well as with n-9 (MUFA), should be sought for.

## METHODS OF ENRICHING OF EGGS WITH OMEGA-3 ACIDS

In order to enrich eggs with omega-3 acids, raw materials rich in unsaturated fatty acids, both of animal and plant origin, are used in feeding layer hens. The materials include fish oil, phytoplankton, crustaceans and sea algae as well as seeds of some oil plants, incl. sunflower, rapeseed, soya, flax or salvia hispanica. Out of them, due to their availability on the market, price, chemical composition and possibility to be used as feed, flax and fish oil deserve special attention.

Flax seeds contain avg. 40% fat, 20–25% protein and 3–10% mucilage [Ansari et al. 2006] and belong to the most highly concentrated sources of unsaturated omega-3 fatty acids found in nature [Caston and Leeson 1990]. The content of α-linolenic acid (ALA) in flax seeds is especially high – according to various sources it varies between 44.6% and 57% of the total content of fatty acids in flax seeds [Ferreira Costa Leite et al. 2011, Morris 2007]. It is well known that this acid is a biological precursor of long-chain acids from the n-3 family (LC-PUFA n-3).

The results of numerous research studies, especially foreign, indicate that flax seeds may be successfully used for the production of eggs enriched with omega-3 acids [Ayerza and Coates 2000, Arshami et al. 2010, Najib and Al-Yousef 2010]. Ferrier et al. [1995] proved that the content of ground flax seeds in feed administered to layer hens (200 g/kg) results in an increased content of α-linolenic acid (ALA) to the level of 527 mg/egg as compared to 28 mg/egg in the control group. Moreover, it results in an increase of the concentration of DHA from 51 to 87 mg/egg, respectively. Laca et al. [2009], who used 2.5% flax seed oil in a typical feed for layer hens, obtained an increased content of EPA and DHA, up to the level of 0.32 and 3.62 mg/g yolk as compared to the content of 0.15 and 1.54 mg/g in the control group. Weill et al. [2002] provides interesting data as well. When layer hens were administered 5% extruded linseed, the content of n-3 PUFA increased to 258.2 mg/egg, whereas in the control group the content was 67.3 mg/egg. Although the content of n-6 PUFA did not change, the ratio of n-6/n-3 acids decreased 3.6 times, the LA:ALA ratio decreased 5.7 times and the AA:DHA ratio was 3 times lower. The conversion of ALA to long-chain omega-3 acids (mainly DHA) in layer hens is effective, contrary to the conversion in human body where, as already mentioned, it is very limited.

Full profile of fatty acids in yolk of eggs non-enriched and enriched in omega-3 acids using linseed or fish oil is presented in Table 1.

The latest research results show that seeds or oils of such plants as hemp, chia (*salvia hispanca* L.) or camelina (*Camelina sativa*) may be used for feeding poultry. Special attention is paid to the fact of high content of linolenic acid in chia seeds (62.0%) and of linoleic acid in hemp seeds (53.9%) (Table 2).

Feeding layer hens with feed enriched with flax or with the seeds or plant oils mentioned above brings about beneficial conversion of ALA to EPA and DHA in the layer hens their building-up in the yolk. Some authors show, however, that EPA and DHA are more valuable biologically than ALA and that is why it is more desirable to enrich food products with these acids [Ollis et al. 1999, Simopoulos 2002].

The easiest method to obtain eggs enriched with DHA and EPA is through adding fish oil, rich in both acids, to the hens' diet. The composition of fatty acids in some fish oils is presented in Table 3. As can be observed, they differ significantly, mainly in the content of LA, ALA, EPA and DHA. It is well known that oils from fish from cold seas contain the highest amounts on n-3 PUFAs. Freshwater fish are characterized by a different profile of fatty acids [Grela et al. 2010]. Thus, the quality of fish oils may be different. Moreover, as they easily undergo the processes of oxidation [Dobrzański et al. 2002], their suitability for feeding purposes may also differ [Koreleski et al. 1994, Usydus 2005, Woods and Fearon 2009].

Table 1. Profile of fatty acids in the yolk of eggs not enriched and enriched with omega-3 acids (% of the total content of fatty acids)

Tabela 1. Profil kwasów tłuszczowych w żółtku jaj niewzbogaconych i wzbogacanych w kwasy omega -3 (% całkowitej zawartości kwasów tłuszczowych)

| Reference Autor / Fatty acid Kwas tłuszczowy | Acc. Trziszka et al. 2011 | | Acc. Sari et al. 2002 | | Acc. Basmacioğlu et al. 2003 | |
|---|---|---|---|---|---|---|
| | Standard diet nie-wzbogacone | Enriched LS+FO wzbogacone LS+FO | Standard diet nie-wzbogacone | Enriched LS wzbogacone LS | Standard diet nie-wzbogacone | Enriched FO wzbogacone FO |
| Myristic C14:0 Mirystynowy | 0.20 | 0.28 | 0.30 | 0.19 | 0.33 | 0.48 |
| Palmitic C16:0 Palmitynowy | 25.0 | 23.1 | 23.45 | 20.38 | 26.33 | 25.80 |
| Palmitoleic C16:1 Palmitooleinowy | nd | nd | 2.83 | 2.32 | 3.19 | 3.57 |
| Stearic C18:0 Stearynowy | 9.07 | 8.20 | 8.67 | 8.99 | 9.01 | 8.22 |
| Oleic C18:1 n-7, n-9 Oleinowy | 43.3 | 44.0 | 38.66 | 36.05 | 38.29 | 39.30 |
| Linoleic LA C18:2 n-6 Linolowy | 17.4 | 15.4 | 20.94 | 22.01 | 17.22 | 16.24 |
| Linolenic ALA C18:3 n-3 Linolenowy | 1.03 | 3.25 | 0.70 | 5.37 | 0.62 | 0.71 |
| Stearidonic C18:4 Stearydonowy | nd | nd | 0.19 | 0.20 | 0.20 | 0.16 |
| Arachidonic AA C20:4 n-6 Arachidonowy | 0.52 | 0.61 | 2.38 | 1.57 | 2.25 | 1.13 |
| Eicosapentaenoic EPA C20:5 n-3 Eikozapentaenowy | 0.01 | 0.19 | 0 | 0.08 | nd | 0.18 |
| Docosapentaenoic DPA C22:5 n-3 Dokozapentaenowy | nd | nd | 0.12 | 0.33 | 0.07 | 0.19 |
| Docosahexaenoic DHA C22:6 n-3 Dokozaheksaenowy | 0.31 | 2.32 | 0.79 | 2.36 | 0.65 | 3.29 |
| Other FA Inne kwasy tłuszczowe | 3.16 | 2.65 | 0.97 | 0.15 | 1.84 | 0.73 |
| Saturated fatty acid (SFA) Nasycone kwasy tłuszczowe | 34.8 | 31.9 | 32.68 | 29.59 | 35.67 | 34.50 |
| PUFA n-3 | 1.35 | 5.76 | 1.80 | 8.35 | 1.54 | 4.53 |
| PUFA n-6 | 18.1 | 16.1 | 23.32 | 23.51 | 19.47 | 17.37 |
| PUFA n-6/n-3 | 13.5 | 2.8 | 13.12 | 2.76 | 12.64 | 3.83 |

LS – linseed – siemię lniane, FO – fish oil – olej rybny, Nd – not detected – nie wykryto

Table 2. Content of the main fatty acids in the seeds and oils of some plants (% of the total content of fatty acids)
Tabela 2. Zawartość głównych kwasów tłuszczowych w nasionach i olejach niektórych roślin uprawnych (% całkowitej zawartości kwasów tłuszczowych)

| Fatty acid / Kwas tłuszczowy<br>Source / Surowiec | Palmitic C16:0 Palmitynowy | Stearic C18:0 Stearynowy | Oleic C18:1 n-9 Oleinowy | Linoleic LA C18:2 n-6 Linolowy | Linolenic ALA C18:3 n-3 Linolenowy | Reference Autor |
|---|---|---|---|---|---|---|
| Rapeseed oil<br>Olej rzepakowy | 6.1 | 2.3 | 56.0 | 24.2 | 6.5 | Fröhlich and Rice 2002 |
| Soya oil<br>Olej sojowy | 11.4 | 4.1 | 22.3 | 53.5 | 7.0 | Glasser et al. 2008 |
| *Camelina sativa* oil<br>Olej z lnicznika siewnego | 5.4 | 2.6 | 14.3 | 14.3 | 38.4 | Fröhlich and Rice 2005 |
| Linseed<br>Siemię lniane | 5.4 | 4.2 | 21.2 | 17.0 | 50.9 | Laca et al. 2009 |
| Hemp seed<br>Nasiona konopi | 6.9 | 2.7 | 10.5 | 53.9 | 24.6 | Gibb et al. 2005 |
| Chia seed<br>(*Salvia hispanica* L.)<br>Nasiona szałwii hiszpańskiej | 6.5 | 2.9 | 7.2 | 20.3 | 62.0 | Ayerza et al. 2000 |

Table 3. Percentage content of the main fatty acids in feed fish oil (authors' own modification)
Tabela 3. Zawartość głównych kwasów tłuszczowych w paszowym oleju rybnym w % (w modyfikacji własnej autorów)

| Reference Autor<br>Fatty acid<br>Kwas tłuszczowy | Acc. Laca et al. 2009* według Laca i wsp. 2009 | Acc. Dobrzański et al. 2002** według Dobrzański i wsp. 2002 | Acc. Palmquist and Griinari 2006*** według Palmquist i Griinari 2006 |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Myristic C14:0<br>Mirystynowy | 3.41 | 3.19 | 8.26 |
| Palmitic C16:0<br>Palmitynowy | 19.10 | 1.70 | 19.52 |
| Palmitoleic C16:1<br>Palmitooleinowy | 6.33 | 2.14 | 11.66 |
| Margaric C17:0<br>Margarynowy | 0.87 | 0.26 | – |
| Margaroleic C17:1<br>Margarooleinowy | 1.05 | – | – |
| Stearic C18:0<br>Stearynowy | 5.14 | 1.82 | 3.82 |
| Oleic C18:1 n-7, n-9<br>Oleinowy | 18.2 | 41.03 | 8.03 |
| Linoleic LA C18:2 n-6<br>Linolowy | 2.37 | 11.74 | 1.30 |

Table 3 cont.
Tabela 3 cd.

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Linolenic ALA C18:3 n-3<br>Linolenowy | 0.86 | 7.30 | 1.45 |
| Gadoleic C20:1<br>Gadoleinowy | 3.72 | – | – |
| Eicosadienoic C 20:2<br>Eikozadienowy | 2.99 | 0.29 | 2.72 |
| Arachidonic AA C20:4 n-6<br>Arachidonowy | 2.16 | 0.14 | – |
| Eicosapentaenoic EPA C20:5 n-3<br>Eikozapentaenowy | 6.52 | 3.98 | 12.04 |
| Docosapentaenoic DPA C22:5 n-3<br>Dokozapentaenowy | – | 0.17 | 2.39 |
| Docosahexaenoic DHA C22:6 n-3<br>Dokozaheksaenowy | 20.8 | 5.88 | 10.11 |
| Other FA<br>Inne kwasy tłuszczowe | 6.96 | 4.53 | 18.7 |
| Saturated fatty acid (SFA)<br>Nasycone kwasy tłuszczowe | 30.23 | 17.28 | 45.68 |
| Unsaturated fatty acid (UFA)<br>Nienasycone kwasy tłuszczowe | 69.77 | 82.72 | 54.32 |

* cantabrian blue fish oil – olej z kantabryjskiej ryby niebieskiej
** oil from post-processing fish waste – olej z końcowego etapu przetwórstwa odpadów rybnych
*** menhaden fish oil – olej z menhadena

Hargis et al. [1991], who used 3% fish oil in the diet of layer hens, obtained eggs containing n-3 PUFAs at an amount of over 180 mg/egg (EPA and DHA) and Meluzzi et al. [2000] obtained 143.70 mg/egg DHA and 19.53 mg/egg EPA at the same amount of oil. De Carvalho et al. [2009], who used 2.4% fish oil in feeding Hisex White layer hens (360 mg DHA/100 g feed), obtained the content of DHA in an egg at the level of 187.91 mg DHA/yolk, and the total amount of polyunsaturated fatty acids (PUFAs) was 218.22 mg/yolk. The introduction of fish oil containing 110 g/kg EPA and 90 g/kg DHA at an amount of 60 g/kg to the diet of the layer hens made it possible to obtain eggs containing avg. 150–200 mg DHA/egg and 45–60 g EPA/egg [Gonzalez-Esquerra and Leeson 2001]. Csuka et al. 2008 introduced only 0.5% of refined fish oil to typical feed mixture and obtained an increase of total content of PUFAs in egg yolk from 11.24 to 12.28%. The content of DHA increased by 350%, as compared to the control group. Attention should also be paid to the study by Ceylan et al. [2011], who used fish oil in amount of 3.0% in a diet of ATE-K hens (brown eggs). In eggs yolk they obtained 3.16 % DHA (% of total FA) when compared to 1.71% (sunflower oil) or 1.64% (rapeseed oil). Plant oils constituted also 3% in feed mixture.

The sparse Polish research results are also worth analysing. Studies on the influence of fish oil added to the feed administered to layer hens on the profile of fatty acids in eggs were conducted at the National Research of Animal Production in Krakow. Over twofold increase of the content of n-3 acids in yolk lipids without negative influence on the taste and flavor of boiled eggs was obtained. For example, the content of DHA in a medium-sized egg was increased from 53 mg to 146 mg and the n-6/n-3 ratio decreased from 7.9 in the control eggs to 4.9 [Koreleski and Świątkiewicz 2008]. The data from the National

Marine Fisheries Research Institute in Gdynia show that the addition of 2 and 4% of fish-mineral concentrate to standard feed administered to layer hens helps decrease the n-6/n-6 ratio in egg yolks over two times and that the first effects may be observed as early as after 2 weeks of feeding hens mixture with the concentrate [Usydus 2005].

Such sea organisms as algae (micro- and macro-algae), esp. those from cold waters, have recently found many applications in farming [Van Ginneken et al. 2011] and feeding farm animals [Sardi et al. 2006, Woods and Fearon 2009, Kupczyński et al. 2011]. Algae contain many nutrients, including omega-3 family fatty acids [Goecke et al. 2010, Chojnacka 2009]. However, the profile of fatty acids is very diversified, e.g. micro-algae *Nannochloropsis oculata* or macro-algae *Palmaria palmate* do not contain DPA or DHA acids at all (Table 4). On the other hand, they may contain large amounts of carotenoids, including lutein, zeaxantin, cantaxantin and β-carotene, which effectively improve the colour of egg yolk. Fredriksson et al. [2006] stated that it is possible to obtain even 4 times more carotenoids (than in the control group), as lutein and zeaxantin constituted as much as 37 mg/kg yolk, incl. 22.0 mg/kg. This, however, requires addition of as much as 20% of pulverised microalgae to the feed mixture. The use of this component in feed for poultry is not complicated technologically, as they are in a form of powder and easily mix with other feed components.

Table 4. Content of the main fatty acids in marine algae (authors' own modification)
Tabela 4. Zawartość głównych kwasów tłuszczowych w algach morskich (w modyfikacji własnej autorów)

| Fatty acid<br>Kwas tłuszczowy | Marine algae – Algi morskie | | |
|---|---|---|---|
| | Algae*<br>Algi | Microalgae**<br>Mikroalgi | Macroalgae***<br>Makroalgi |
| Myristic C14:0 – Mirystynowy | nd | 3.5 | 0.68 |
| Palmitic C16:0 – Palmitynowy | 18.0 | 17.9 | 3.50 |
| Palmitoleic C16:1<br>Palmitooleinowy | nd | 19.0 | 0.20 |
| Stearic C18:0<br>Stearynowy | 0.33 | 0.7 | 0.13 |
| Oleic C18:1 n-7, n-9<br>Oleinowy | 0.11 | 5.6 | 0.48 |
| Linoleic LA C18:2 n-6<br>Linolowy | 0.31 | 7.4 | 0.13 |
| Linolenic ALA C18:3 n-3<br>Linolenowy | 0.25 | 6.7 | 0.31 |
| Arachidonic AA C20:4 n-6<br>Arachidonowy | nd | 3.7 | 0.33 |
| Eicosapentaenoic EPA C20:5 n-3<br>Eikozapentaenowy | 1.28 | 37.1 | 8.34 |
| Docosapentaenoic DPA C22:5 n-3<br>Dokozapentaenowy | 6.95 | nd | nd |
| Docosahexaenoic DHA C22:6 n-3<br>Dokozaheksaenowy | 17.1 | nd | nd |

Nd – not detected – nie wykryto
* *Schizochytrium sp*. – acc. Sardi et al. (2006) – g/100 g of total FA
** *Nannochloropsis oculata* – acc. Fredriksson et al. (2006) – % of total FA
*** *Palmaria palmate* (Nord Atlantic) – acc. Van Ginneken et al. (2011) – mg FA per g/DM

**THE PROBLEM OF FISH FLAVOUR AND TASTE**

The use of fish products and flax in the diet of layer hens is often related to the problem of "fish smell" of the eggs as well as to the occurrence of strange taste which, at present, is the biggest, undesirable effect related to using these materials [Parpinello et al. 2006]. Research results show that it is due to the oxidation of polyunsaturated fatty acids [Van Elswyk 1997, Cherian et al. 2008], although some authors suggest that these undesirable organoleptic features of egg material result from the oxidation of fatty and non-fatty substances contained in feed [Leskanich and Noble 1997]. That is why using natural or synthetic antioxidants, e.g. vitamin E in the diet of layer hens, is frequently practiced in the production of enriched eggs [Gonzalez-Esquerra and Leeson 2001, Cherian 2008, Shapira 2010]. Adding this vitamin at the level of 200 mg/kg feed to the diet of hens gives the desired antioxidative effect, but does not fully prevent the worsening of sensory features of eggs [Galobart et al. 2001, Laca et al. 2009]. Alfalfa, more and more widely used in feeding poultry, is a very good source of antioxidants. However, its influence on reducing the occurrence of undesired flavour of eggs requires further studies [Bubel et al. 2010]. Selenium (Se) contained in feed also has some antioxidative features, but the results of studies in this matter are not unequivocal and it is also known that an increased level of selenium may be toxic for birds [Surai 2002].

The negative sensory features of eggs do not always reveal after culinary treatment or pulverising. It depends on the amount and the quality of the sources of omega-3 acids in the diet of layer hens and also on the content of other feed components (e.g. post-extraction meal). Generally, the undesirable effects of using flax or/and fish oil were observed only when they were introduced to the diet of layer hens at the amounts higher than 5% in case of flax and 1.5% in case of fish oil [Surai and Sparks 2001]. It is also worth adding that some genetic lines of layer hens which lay brown eggs do not have oxidase – an enzyme breaking down trimethylamine (TMA) which accumulates in egg matter and is responsible for unpleasant flavor and taste. This is especially frequent in case of feeding hens with rapeseed feed (meal, pomace, oil) [Ward et al. 2009].

There are several methods of preventing the negative organoleptic features of eggs or egg material. There were attempts at deodorizing fish oil by removing ketones and compounds rich in benzene as they play an important role in the creation of fish flavor. Unfortunately, the results were not satisfactory because the metabolism of feed fats is chemically complicated and there are numerous factors influencing the sensory features of eggs and the features of food products containing them [Gonzalez-Esquerra and Leeson 2001, Kassis et al. 2010]. Although the use of fish oil in the form of microcapsules (at relatively low content in diet) may help decrease the fish flavour in eggs, it increases the price of this feed material and the costs of egg production [Lawlor et al. 2010].

Ayerza and Coates [2001] stated that adding a combination of flax and chia (rich in ALA) to the diet of layer hens, maintaining the level of flax below 5% in the feed mixture, brings about an increased content of ALA in egg yolk without the unfavourable influence on the flavor and taste of eggs. The combination of fish oil with humine and aluminosilicate materials significantly reduces the unpleasant taste and flavour of eggs under the condition that the dose of fish oil is not too high [Usydus 2005]. There is little data available on the influence of the addition of sea algae on the organoleptic features of eggs, although the research results indicate that it does not result in worsening of these important quality features [Fredriksson et al. 2006].

Experimental research studies are now conducted to increase the content of n-3 PUFAs in hen and quail eggs to the maximum using products of marine origin and selected oil plants and, at the same time, limiting the negative sensory features (fish flavour and taste).

It should be stated in the summary that there are currently practical possibilities to obtain hens (and quail) eggs enriched in omega-3 polyunsaturated fatty acids by an application of feed fats in laying poultry feeding (fish oils and from oilseed crops) or special supplements containing marine organisms meals (algae) or some plant seeds (cannabis, Salvia hispanica). The consumption usefulness of these eggs is conditioned by sensory features (taste, smell) which depend on an amount of feed additives used in feeding dose and presence of substances limiting negative quality features of eggs. They include antioxidants (selenium, vitamin E) and some mineral sorbents (humic, aluminosilicates). An open problem, not analysed by the authors, is an economic effectiveness of enriched eggs production and their technological usefulness in processing.

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## WZBOGACANIE JAJ KURZYCH W WIELONIENASYCONE KWASY TŁUSZCZOWE – ASPEKTY FIZJOLOGICZNE I ŻYWIENIOWE

**Streszczenie.** Omówiono biologiczną rolę wielonienasyconych kwasów tłuszczowych z grupy omega-3, a więc α–linolenowego (ALA, C18:3 n-3) eikozapentaenowego (EPA, C20:5 n-3), dokozapentaenowego (DPA, C22:5 n-3) i dokozaheksaenowego (DHA, C22:6 n-3). Przedstawiono źródła ich występowania (organizmy morskie i niektóre nasiona roślin

oleistych) możliwości zastosowania paszowego u drobiu oraz wzbogacania jaj kurzych w te kwasy. Omówiono problemy tzw. rybiego posmaku i zapachu jaj oraz sposoby ograniczania tej negatywnej cechy sensorycznej.

**Słowa kluczowe:** jaja, wielonienasycone kwasy tłuszczowe omega-3, pasza

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# HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES OF HEPATOCYTES IN RED-EARED TURTLES (*TRACHEMYS SCRIPTA ELEGANS*) DURING HIBERNATION AND AFTER AROUSAL

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**Abstract.** This research was conducted on 22 red-eared sliders during hibernation and after arousal. The material came from Poznan Zoo (Poland). Species affiliation was established on the basis of the Turtles of the World key. The examined sliders were of a similar age. The histological, histochemical and ultrastructural studies were conducted during hibernation and after arousal. Additionally analysis of liver elemental composition (C, $O_2$, Na, Ca, Al., P, Mg and S) was performed. The results showed morphological and ultrastructural liver changes, related to hibernation process concerning shape and size of hepatocytes as well as morphology and location of cell organelles. The conducted elemental analyses revealed during hibernation the reduction of the phosphorus levels up to70–80% while after arousal there was distinct increase of oxygen, sodium and aluminum concentration noticed.

**Key words:** red-eared turtles, liver, hepatocytes, hibernation

## INTRODUCTION

There have been a number of scientific studies into the phenomenon of hibernation among vertebrates [French 1985, Wang 1987, Constanzo and Claussen 1990, Claussen et al. 1990]. The liver is usually considered the crucial organ in metabolic processes during hibernation [Costanzo and Claussen 1990, Hastings and Ebling 2006]. In research on *Testudo graeca* it has been shown that hibernation mainly influence the amount and location of cell organelles and reserve materials within hepatocytes. Moreover it was indicated that in hibernating animals a certain percentage of hepatocytes exhibit the features of degeneration (shrinkage of liver cells and cytoplasm vacuolization). Morphological



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changes during hibernation in the chosen parenchymal organs, including the liver, in vertebrates were also presented by: Hemmings and Storey [2000]. The aim of this study was not only to describe the morphology, ultrastructure and morphometry of the hepatocytes during hibernation and after arousal but also to examine the biochemical properties of turtle liver and its changes connected with hibernation. Moreover an attempt has been made at determining the topography of chemical elements (mapping) of the hepatocytes which would allow for a precise definition of which microelements play a fundamental role in process of hibernation and what their dynamics are.

## MATERIALS AND METHODS

For the research 22 liver specimens from red-eared turtles were used. The turtles, collected in the years 2002–2006, came from Poznan Zoo, where proper animal welfare and sanitary conditions were guaranteed. The animals were maintained in open pools. From the beginning of October 2006 the turtles were given increasingly less food in order to bring them into a lethargic state. The turtles hibernated in conditions close to natural ones. All the animals were placed in separate boxes of a volume of 100 l (1 box for 3 turtles) filled with a mixture of leaves and peat. Deep hibernation was obtained at the beginning of November, when the temperature was 8°C. The animals were hibernated for a period of 4 months. During that period, they were regularly sprinkled every 2 weeks with 8°C running water. Among the animals under analysis the first signs of awakening were observed in the middle of February 2007 when the temperature increased to 10°C. The animals were moved to the laboratory with a room temperature of 21–22°C and they were euthanized using Morbital (Biowet, Puławy) given intraperitoneally. The samples of the liver were collected within 25 minutes of delivery to the laboratory. All procedures including euthanasia protocols were approved by the Local Ethics Committee. The animals were all males of a similar age (8 years old). The liver samples were collected from the left liver lobe. All the specimens were weighed *post mortem* on a analytical scale (Siemens) with an accuracy of up to 0.01g.

The animals were divided into two groups:

1. Group I – individuals that were euthanized after arousal (June to July 2007)
2. Groups II – individuals that were euthanized during hibernation (January to February 2007)

***Light microscopy.*** Liver samples designated for observation in a light microscope were rinsed in saline solution (0.9% NaCl) and then fixed in a 10% formaldehyde solution for at least 48h at room temperature. Next, sections were dehydrated in alcoholic series (from 60% alcohol to absolute alcohol). The dehydrated material was paraffin embedded. Such samples once prepared were cut with the use of a rotary microtome HM 340 E (Microm International, Walldorf, Germany). In the histological examination the following staining method was used: PAS (by Schiff's reagent).

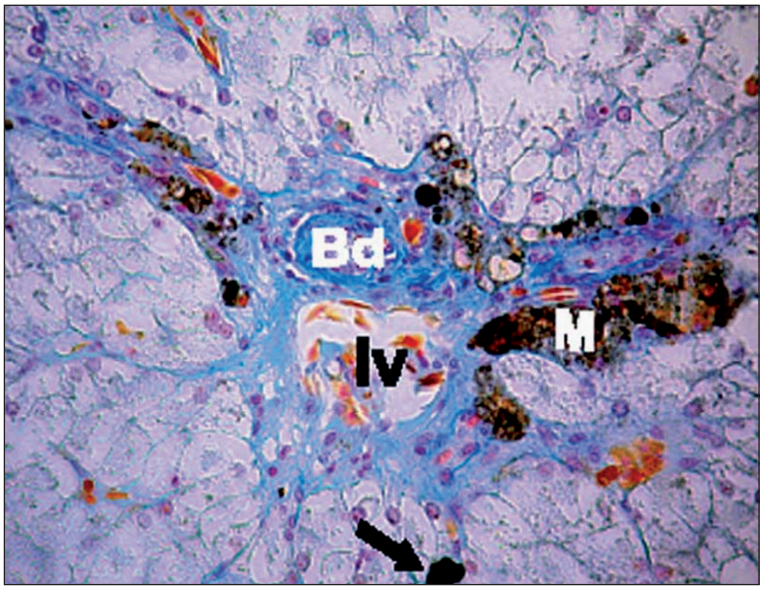
***Transmission electron microscopy* (*TEM*).** The material designated for observation in a transmission microscope was fixed in 2.5% glutaraldehyde on a 0.1 M phosphate buffer of pH 7.4 at room temperature for at least 48 h, and then rinsed in a phosphate buffer (30 min). Next, the material was postfixed in 4% $OsO_4$ for 2 h. After repeated rinsing in a phosphate buffer the liver fragments were dehydrated in alcoholic series (from 60% alcohol to absolute alcohol) (15 min each) and 100% acetone (for 1 h). The dehydrated material was infiltrated in a mixture of acetone + epon (1:1) for 12 h and embedded in Epon 812 epoxy resin. Epon blocks were cut with the use of a ultramicrotome MTX (Leica). The preparations were examined in a Tesla BP 500 transmission electron microscope.

***Analysis of liver chemical composition***. The examined material was fixed in 2.5% glutaraldehyde (48 h), then rinsed in a phosphate buffer and dehydrated in alcoholic series (40–100%). Next, the material was dried and stuck on tables. The elemental analysis was conducted with the use of a Leo 435 VP (Zeiss) scanning microscope with accelerating voltage from 15 to 20 kV, coupled with microroentgen analyzer ROENTEC (Oxford).

***Morphometric and statistical analysis***. Morphometric analysis was conducted with the use of the Medium program coupled with a light microscope (Leica). The total surface of hepatocytes was estimated on a working surface of preparations 0.5 cm x 0.5 cm. During the statistical analysis the following parameters were considered: total surface of liver cells and total levels of C, $O_2$, Ca, Na, Al, P, Mg and S. Statistical research was prepared with the use of the statistical Statistica 6.0 (StatSoft) programme. Mean values and standard deviations of percentage of elements were determined, as well as the surface area of hepatocytes. All the measurements were performed on turtle from group I and II. Next, the t-Student test was used for independent samples, since the research concerned various specimens at two different stages of the yearly cycle. The analysis was performed to define whether the percentage elemental composition of the turtle liver and change of liver cell surface reveals statistically significant differences during hibernation and after arousal.

## RESULTS

***Histological assessment of liver after arousal.*** The liver was surrounded by a thin connective tissue capsule. The liver cells were of polygonal or pyramidal shape. Nuclei with prominent nucleoli within hepatocytes were situated centrally or eccentrically. In most cases only one nucleus occurred with only seldom binucleated cells observed. The hepatocytes in the amount of 4 to 5 were located around bile tracts and concentrated in trabeculae. In the parenchymateous part they had an irregular shape, whereas in the perivascular zone they were located radiantly (Phot. 1). The histological research revealed the presence of glycogen in liver cells located in the area of the whole cytoplasm. In the vicinity of large blood vessels extensive clusters of mononuclear cells were observed (Phot. 1). Additionally, numerous pigment loaded cells with diameters larger than hepatocytes were noticed. They were gathered in clusters or situated more irregularly.

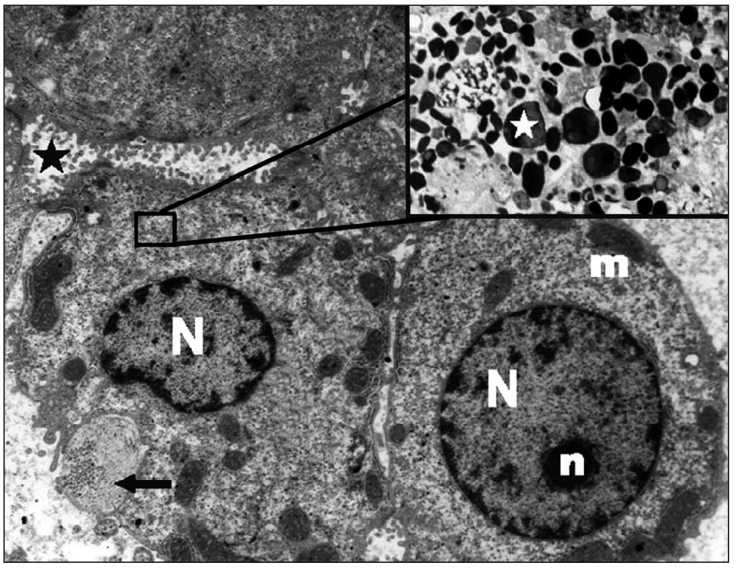



Phot. 1. The fragment of hepatic lobule with interlobular vein (Iv), bile duct (Bd), melano-macrophage (M) and pigment cell (arrow). PAS. Mag 200 X

Fot. 1. Fragment płacika wątrobowego z żyłą międzypłacikową (Iv), przewód żółciowy (Bd), melanomakrofag (M) i komórka barwnikowa (strzałka). PAS. Mag 200 X

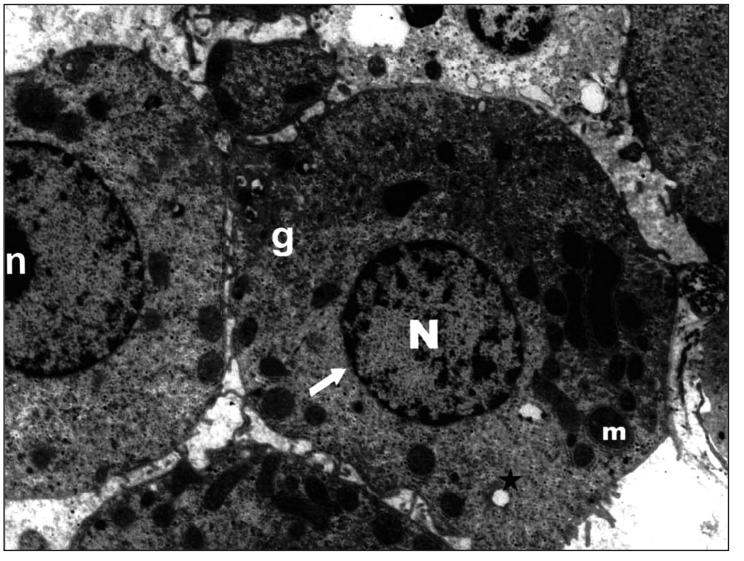
***Ultrastructural research after arousal.*** Research using a transmission microscope showed that hepatocytes had numerous cytoplasmic processes directed towards the perisinusoidal space, however some microvilli were in direct contact with endothelial cells (Phot. 2). In periphery of some hepatocytes membrane-bound vacuolar structures were observed. In nuclear membrane marked nuclear pores were visible. The presence of smooth and rough endoplasmic reticulum as well as glycogen particles was confirmed. In the cytoplasm free ribosomes and irregularly located mitochondria were observed (Phot. 3). Most often they were located near the nucleus and were of oval or longitudinal shape (Phot. 2). In the vicinity of bilary canaliculi the Golgi's system was observed in the form of cisterns and secretory vacuoles. The ultrastructural research also proved the presence of pigment cells. They exhibited the presence of pigment granules of different shapes (round, oval and longitudinal) (Phot. 2). Pigment cells occurred in largest numbers in the perivascular area.

***Histological assessment of the liver during hibernation.*** The liver was surrounded by a thickened connective tissue capsule (Phot. 4). The liver cells were mostly of an irregular pattern. Free spaces filled with tissue fluid occurred between hepatocytes. In the basilar zone of the hepatocyte a nucleus with a poorly visible nucleolus was identified. The liver cells were of poligonal shape and their cytoplasm was acidophilic and vacuolated. Some of the hepatocytes exhibited degenerative changes. Some pigment cells were also observed.



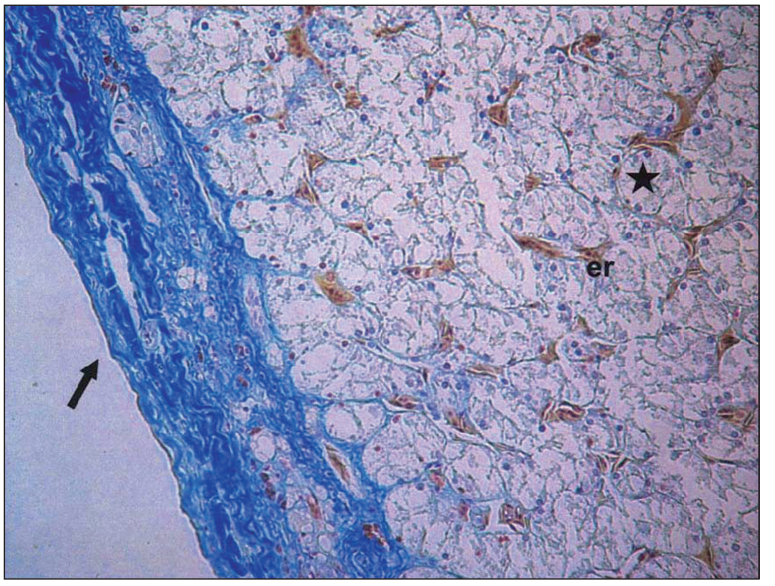

Phot. 2. The hepatocytes and melano-macrophage with visible melanin granules (white *, TEM. Mag 32 000 X) in red-eared turtles. In peripheral zone of phagocyte visible lisosomal vacuole (arrow). In central part of cell nucleus (N), nucleolus (n), mitochondrium (m) and interlobular bile duct were visible (black *). TEM. Mag 10 000 X

Fot. 2. Hepatocyty i melanomakrofagi z widocznymi ziarnami melaniny (biała *, TEM. Mag 32 000 X) u żółwia czerwonolicego. W strefie brzeżnej fagocytu widoczna wakuola (strzałka). W części centralnej hepatocyta widoczne jądro (N), jaderko (n), mitochondrium (m) i przewód żółciowy (czarna*). TEM. Mag 10 000 X




Phot. 3. Red-eared turtles hepatocyte after arousal; nucleus (N), nucleolus (n), nuclear membrane (arrow), mitochondrium (m), glycogen (g). TEM. Mag 6000 X

Fot. 3. Hepatocyt żółwia czerwonolicego po hibernacji; jądro (N), jąderka (n), błona jądrowa (strzałka), mitochondrium (m), glikogen (g). TEM. Mag 6000 X

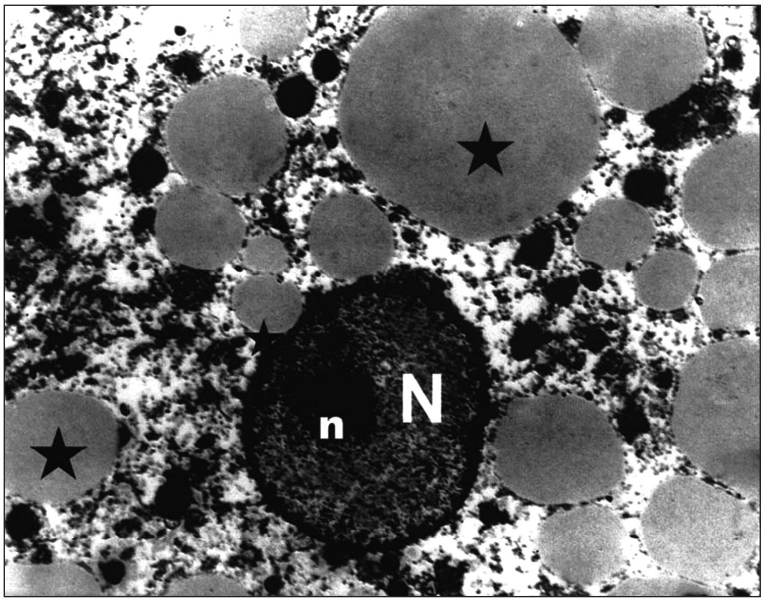



Phot. 4. The clearly thickened connective capsule. Note erythrocytes (er) and hepatocytes (*). PAS. Mag 100 X

Fot. 4. Wyraźnie pogrubiona torebka łącznotkankowa wątroby. Erytrocyty (er) i hepatocyty (*). PAS. Mag 100 X

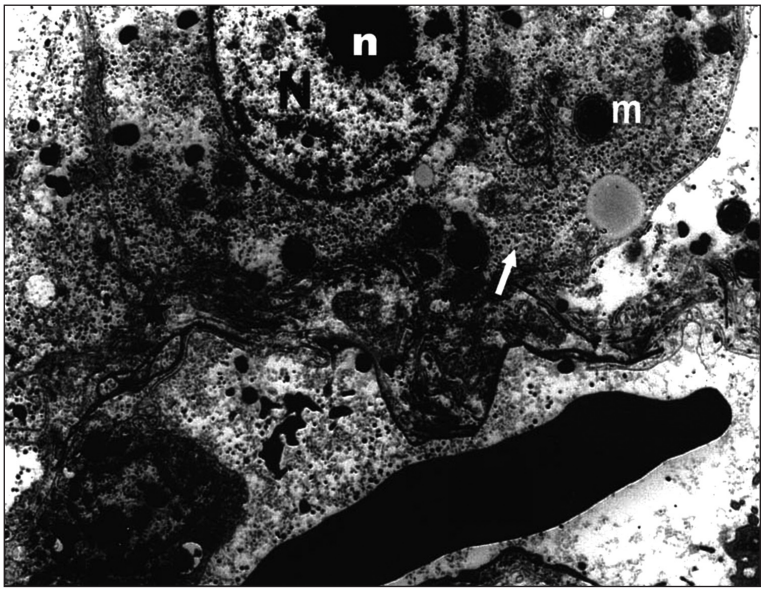
***Ultrastructural research* (*TEM*) *during hibernation.*** Mitochondria occurring in hepatocytes were enlarged and with an evidently round shape. There were a lot of lipid droplets within the cytoplasm of the extended hepatocytes. Focally the cell membrane showed features of damage. In the peri-sinusoidal spaces characteristic cytoplasmic processes of hepatocytes were not evidenced (Phot. 5 and 6).

***Analysis of the liver elemental composition***. An analysis of the following elements was conducted: C, $O_2$, Na, Al, Mg, Ca, S and P. The average value (x) as well as standard deviation of individual analyzed elements concentrations in liver turtles during hibernation and after arousal showed Table 1. The null hypothesis was formulated as follows: $H_0$ – there are no differences in content of the liver elements between specimens after arousal and during hibernation. Whereas the competitive hypothesis was: $H_1$ – there is a difference in content of elements of the turtle liver during hibernation and after arousal. For all the conducted statistical tests it was assumed that the significance level is $p<0.05$. The results obtained for each element were analyzed separately. Statistically significant differences were shown between the examined groups (Table 1). They were indicated with regards to C, P, S and Na content. There were no statistically significant differences in the levels of the following elements: $O_2$, Al, Mg and Ca and Na in the liver before and after hibernation (Table 1). In the second part of the statistical research concerning the change in the liver cells' surface after arousal and during hibernation the t-Student test for independent samples was used. For all the conducted statistical tests it was assumed that the significance level is $p<0.05$. On the basis of the conducted statistical analysis significant decrease of the surface area of liver cells during hibernation was revealed (Diagram 1).




Phot. 5. The fragment of hepatic lobule, visible accumulation of large and small lipid droplets (*) and glycogen in perinuclear zone. Note nucleus (N) and nucleolus (n). TEM. Mag 8000 X

Fot. 5. Fragment płacika wątrobowego, widoczne nagromadzenie małych i dużych kropel tłuszczu (*), glikogen w strefie okołojądrowej. Jądro (N) i jąderko (n). TEM. Mag 8000 X




Phot. 6. The fragment of hepatocyte. Nucleus (N) and nucleolus (n). The hepatocytes cytoplasm with presence of numerous glycogen granules (arrow). Visible mitochondia (m). TEM. Mag 10 000 X

Fot. 6. Fragment hepatocytu z licznymi ziarnami glikogenu (strzałka). Jądro (N), jąderko (n) i mitochondria (m). TEM. Mag 10 000 X

Table 1. The average value as well as standard deviation of individual elements concentrations in liver turtles during hibernation and after arousal

Tabela 1. Średnia oraz odchylenie standardowe pierwiastków chemicznych wątroby u żółwia czerwonolicego w czasie i po hibernacji

| Chemical element Pierwiastek chemiczny | After arousal Po hibernacji | | During hibernation W czasie hibernacji | | p<0.05 |
|---|---|---|---|---|---|
| | average [%] średnia | SD [%] | average [%] średnia | SD [%] | |
| **C** | 60.02 | 10.1 | 47.73 | 9.88 | 0.0145 |
| **$O_2$** | 35.18 | 11.23 | 44.98 | 9.05 | 0.0652 |
| **Na** | 0.66 | 0.37 | 1.26 | 0.97 | 0.0399 |
| **Al.** | 0.46 | 0.62 | 1.77 | 2.41 | 0.1318 |
| **P** | 1.92 | 0.62 | 0.98 | 0.5 | 0.0017 |
| **Mg** | 0.21 | 0.24 | 0.11 | 0.17 | 0.3766 |
| **S** | 1.15 | 0.46 | 0.56 | 0.38 | 0.0011 |
| **Ca** | 0.36 | 0.08 | 0.08 | 0.08 | 1.0000 |

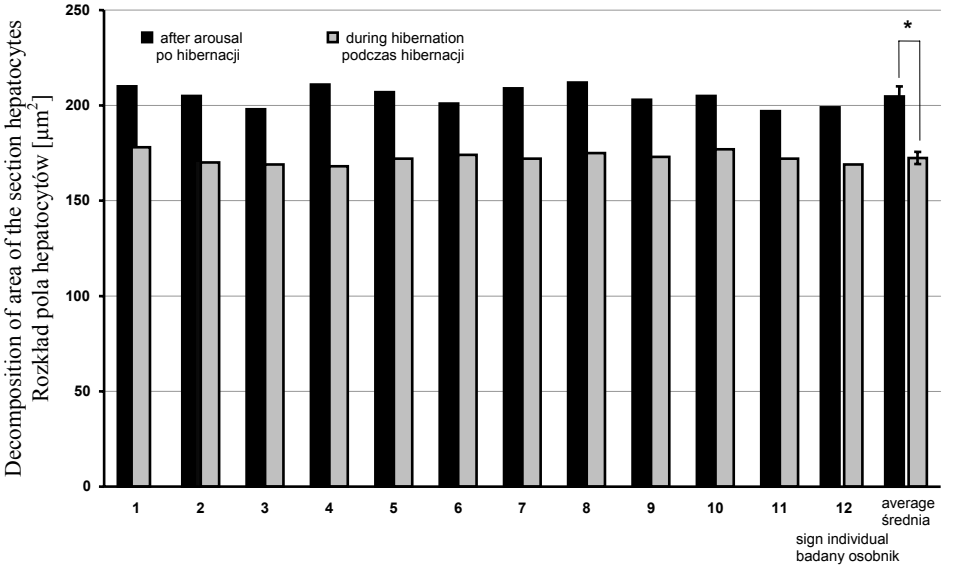



Diagram 1. The value average of hepatocytes surface during hibernation and after arousal

Wykres 1. Średnie wartości powierzchni hepatocytów w czasie hibernacji i po hibernacji

## DISCUSSION

Hibernation is characterized by a considerably lowered metabolic level [Wang 1987, French 1988]. During hibernation, the turtles reach a physiological minimum essential to maintain live activity in unfavorable thermal conditions. This process is characterized by a considerable decrease in body temperature and an inhibition of liver protein synthesis [Malatesta et al. 2001]. The ability to maintain life functions during the freezing of body liquids is a characteristic feature of numerous heterothermic animals [Churchill et al. 1994]. The natural tolerance of freezing is observed in e.g. *Rana sylvatica, Hyla versicolor, H. chrysoscelis, H. crucifer, Pseudacaris triseriata, Hynobius keyserlingi, Terrapene carolina* and also young tortoises *Chrysemys picta* [Constanzo and Claussen 1990]. All these species can survive for a long period in temperatures even lower then 8°C. The liver regulates the metabolism connected with the hibernation process [Hasting and Ebling 2006]. This study reviled morphological, ultrastructural and biochemical changes in the red-eared turtles' liver undergoing during the winter sleep in a form of changes of shape and size of hepatocytes as well qualitative and quantitative features of cells' organelles. Similar changes were observed in other vertebrates by Miscalencu et al. 1978, Hacking et al. 1987. During hibernation reorganization of the liver cell occurs. These changes concern the location of cell organelles and reserve materials in hepatocytes [Hemmings and Storey 2000]. The present study also showed the tendency to hepatocytes shrinkage and regressive changes during hibernation in red-eared turtles. In observations of Greek turtle such regularities were not found which may be explained by alternative environmental or feeding conditions. In the conducted research it was noticed that during hibernation the hepatocytes' nucleus increases in size. At that time also the amount of rough endoplasmic reticulum (RER) decreases and smooth endoplasmic reticulum (SER) increase. In amphibia only the decrease of RER was noticed, whereas no changes concerning the SER was indicated. Morphologic characteristics of the nucleus and cytoplasm in Greek tortoise was the basis for describing two kinds of liver cells. The first one contains dark cytoplasm (dark cells) and the second contains bright cytoplasm and numerous mitochondria (clear cells). The available literature says that in reptiles you can find up to four nucleoli in the cell [Gans and Gaunt 1998]. In the present research it has been established that in red-eared slider usually one nucleolus occurs. The most characteristic feature of the hibernating liver cell are morphologic changes in mitochondria. In reptiles they take different shapes. The most characteristic is the oval shape, however the current research indicates that they can take a longitudinal shape. Many authors describe morphologic and ultrastructural changes within mitochondria during hibernation [Zimny and Moreland 1968, Fonda et al. 1983, French 1985, Wang 1987]. They number and size are strictly connected with oxygen consumption especially during the winter sleep [Gans and Gaunt 1998]. The strategy of hibernating and food availability during hibernation can have an unquestionable influence on the morphology and topography of mitochondria in the liver cell. In conducted study mitochondria occurring in hepatocytes were enlarged and with an evidently round shape. Heterothermic animals usually store fat in subcutaneous tissues. The fat plays the role of thermal insulation during winter and is also energy storage. Reptilia, like heterothermic animals, adopted another strategy for survival in low temperatures, thus they collect a considerably smaller amount of fat in subcutaneous tissues [Girons and Duguy 1992]. The ultrastructural study of red-eared slider liver with respect

to fat content after arousal demonstrated that fat droplets deposited in hepatocytes were present only sporadically. They occupied a considerably smaller area and were arranged in the cell regularly not destroying the structures of the cell. In the case of hibernated turtles in turn, fat droplets were spherical structures of a diameter of a few microns, and occupied almost all the area of liver cell moving the cell nucleus to the peripheral area. This phenomenon in mammals is referred to as micro- and macro-functional fatty degeneration of the liver. This process should be distinguished from adipose liver degenerative which is connected with damage of the cell structures. Functional accumulation of the fat droplets in the liver in turn is a result of properly progressing processes of fat transformation. Such a state is characteristic for the Chelidae, *Phrynops hilarii* [Da Silva and Migliorini 1990]. The liver is also the main storage of glycogen [about 10% of organ mass]. In the context of hibernation a significant role is played by processes connected with accumulation of glycogen and its utilisation during waking from the winter sleep. The conducted research confirm significant accumulation of glycogen during hibernation in the red-eared slider liver. The role of glycogen in the process of the winter sleep and initial stages of waking from the sleep are described by: Storey et al. [1990], Hemmings and Storey [2000], Warren et al. [2006], Mehrani and Storey [1995]. Those results are convergent with the studies concerning turtle nestlings *Chrysemys picta marginata* [Hemmings and Storey 2000]. It was shown that is accumulated in the liver 0.88% mg/g of glycogen in the last winter sleep stages. During hibernation many functional and morphologic changes occur within the liver cell [Barni et al. 2002]. In response to the low temperature of the environment, which lasts for a few months, and lack of food also changes in location of melanin in the liver cell occur. Melanin can occur in the liver in two forms: as a component of pigment cells and within macro-melanophages [Barni et al. 2002]. Pigment cells in turns occurred in two morphologic forms. They formed numerous aggregates occurring in all areas of the hepatic lobule or are spotted individually in the surroundings of large blood vessels [Rund et al. 1998]. Pigment cells and melanomacrophages occurred in a similar quantity both after arousal and during hibernation in red-eared slider. The conducted elemental composition of the liver showed that during hibernation there is a decrease of phosphorus level. In our opinion it is caused by significant energetic expenditure assigned for maintenance of basic life processes during the winter sleep. A similar decrease was observed with regards to sulphur and calcium. In our opinion, the reason for this is the involvement of the above mentioned elements in stabilization processes of hepatocytes and connective-tissue stroma of the liver. Also a decrease in the carbon level is observed, which in fact may be explained by reduction and degradation of both liver cells and connective-tissue stroma. Other elements such as oxygen, sodium or aluminum reveal a rising trend during hibernation. During hibernation most vital processes are inhibited, however the increase of oxygen and sodium contents can be a specific guarantee for obtaining balance in the very first period of metabolic activity. Moreover liver supports the oxidation-reduction processes. Therefore we think that the increase in oxygen content, seems to be absolutely justified.

In conclusion conducted study confirms multilateral differences at morphological, ultrastructural and biochemical level between liver samples collected from red-eared turtles during hibernation and after arousal. Observed differences point at crucial role of liver in the course of hibernation process. Moreover gathered findings may serve as a valuable background for veterinary practitioners in the course of turtles treatment and breeding protocols in small animal practice.

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## HISTOLOGICZNE I ULTRASTRUKTURALNE ZMIANY W WĄTROBIE U ŻÓŁWIA CZERWONOLICEGO (*Trachemys scripta elegans*) W CZASIE I PO HIBERNACJI

**Streszczenie.** Badania wątroby przeprowadzono na 22 osobnikach płci męskiej żółwia czerwonolicego w czasie hibernacji i po hibernacji. Materiał ten pochodził z Ogrodu Zoologicznego w Poznaniu. Gatunek żółwia określono na podstawie klucz Turtles of the Word [Ernst, Barbour 1989]. Wszystkie badane osobniki były w jednym wieku (8 lat). Przeprowadzono badania histologiczne, histochemiczne i ultrastrukturalne wątroby. Wykonano również analizę chemiczną składu pierwiastkowego wątroby (C, $O_2$, Na, Ca, Al., P, Mg i S). Badania morfologiczne i ultrastrukturalne wątroby wykazały zmiany w kształcie i wielkości hepatocytów w czasie hibernacji, jak również stwierdzono zmiany w budowie i rozmieszczeniu organelli komórkowych. Przeprowadzona analiza chemiczna składu pierwiastkowego wątroby podczas hibernacji wykazała spadek poziomu fosforu o 70–80%, podczas gdy zaobserwowano wzrost koncentracji tlenu, sodu i aluminium.

**Słowa kluczowe:** żółw czerwonolicy, wątroba, hepatocyty, hibernacja

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# COMPARISON OF METHODS OF FERTILE OESTRUS INDUCTION IN FEMALE GOATS OUTSIDE THE PHYSIOLOGIC BREEDING SEASON

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**Abstract.** The objective of this study was to compare the efficiency of different estrus induction protocols in anoestrus goat. Does were assigned to seven groups (A-G). In the group A, the animals (n=25 ) received intravaginal sponges containing 30 mg of flugestone acetate FGA (Chronogest, Intervet) for 12 days and i.m. injection of 500 I.U. of PMSG (Folligon, Intervet) two days before sponges withdrawal. In the groups B(n=10), C (n=10), D (n=10) females received 12,5 mg progesterone (Progesteronum, Polfa Warszawa S.A.) per animal in i.m. injections respectively for 6, 12 or 2 days. Females from groups E (n=10) and F (n=10) received altrenogest (Regumate, Intervet) p.o. for 6 or 12 day. At the end of progestagen treatment the animals from group B, C, D, E, F received 500 I.U. of PMSG i.m. The goats from control group G (n=10) did not received any treatment. During the experiment, a significant difference was found in the effectiveness of the oestrus induction among applied methods. Oestrus occurred in 72% of females from group A, 60% from group B, 100% from group C and 20 % of females from group G. Oestrus was not observed in any female of group D, E and F. We concluded that the duration of the progestagen treatment significantly influenced the efficiency of the oestrus induction protocol in anoestrus goats.

**Key words:** goats, anoestrus, estrus induction, progesterone



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## INTRODUCTION

The problem of induction of oestrus in small ruminants outside the season was a subject of numerous studies. However, a majority of them refers to ewes and to the application of intravaginal sponges (currently commercially unavailable in many countries) [Morand-Fehr and Lebbie 2004]. The problem of oestrus stimulation in goats was not thoroughly studied due to common opinion about the close relationship between these species hence the similarity of reproductive processes. This opinion were verified in research made by Ginther and Kot [1994] who pointed out the significant differences in reproduction physiology of both species. This was also confirmed in experiments carried out by Schwarz and Wierzchoś [2002, 2004]. These authors drew the attention to the fact that despite an extremely important feature of reproduction seasonality common for the goat and the sheep, also important differences between both species can be observed (e.g. significantly lower reduction of the folliculogenesis rate in goats in the anoestrus period).

The induction of oestrus outside the reproductive season in goats may be based on the light program manipulation, an administration of melatonin, or programs based on progestagen administration [Fonseca et al. 2005, Freitas et al. 1997, Greyling and Nest 2000, Motlomelo et al. 2002, Papachristoforou et al. 2007, Zarkawi et al. 1999]. In a case of progestagen programs, the modifications refer both to the kind of an active substance, the dose as well as the duration of hormonal stimulation [Daniel et al. 2001, Freitas et al. 1996, 1997, Wildeus et al. 2003, Vinoles et al. 2001, Zarkawi et al. 1999].

The objective of the studies was to compare the effectiveness of several methods of induction of fertile oestrus in goats outside the reproductive season. The use of different substrances, different way of administration and various duration of the treatment was analyzed.

## MATERIALS AND METHODS

The researches were carried out in April in the goat farm situated near Wrocław, in Poland. All investigated animals were between their first and third lactation, were kept in similar conditions and were under the same feeding protocol. A total of 85 goats of local breed were divided randomly in to seven groups(A-G).

The animals from group A received a Chronogest sponges (Intervet) containing 30 mg flugestone acetate intravaginaly (n=25) for a period of 12 days. Two days before sponges removal, a 500 U.I. PMSG was administrated intramusculary. Females from group B (n=10) received a 12,5 mg per animal of progesterone (Progesteronum, Polfa) in intramuscular injection for 6 days, and 500 I.U. PMSG (i.m.) on the 7th day. In group C (n=10) progesterone was administered according to the similar pattern for 12 days with PMSG injection on 13th day, while in group D (n=10) only 2 injections of progesterone in the same dose were given in combination with PMSG injection on the 3th day. To the females from group E (n=10) suspension containing altrenogest (Regumate, Intervet) in a dose 0,0044 mg/kg were given per os with the use of syringe. This treatment was continued for 12 days while in the animals from group F (n=10) it was carried out only for 6 days. The injection of 500 I.U. of the PMSG was performed in the animals from group E and

F on the day 13th and 7th respectively. Group G (n=10) consisted of females receiving no medicines. In the experiment, groups A (intravaginal sponges) and G (no hormonal stimulation) were the control groups. Application of the intravaginal sponges is currently the basic method of oestrus induction and synchronization in small ruminants that is used worldwide. Its effectiveness was confirmed in numerous studies and therefore the method was our reference point for other investigated methods [Freitas et al. 1996, 1997].

In groups B, C, D, E and F the progesterone or progestagens were always administered to the animals at the same part of the day (during a morning milking). In order to determine the progesterone level in serum, from the animals from group B, C, D blood was taken from the jugular vein on the day 0, 1, 6, 9, 11 and 13. Plasma was separated by centrifugation for 10 min at 2000g and was stored at -20 ºC until assayed for progesterone. Determination of progesterone was performed with the use of the EIA method (enzyme immunoassay-Immulite 2000 Progesterone, DPC, USA) [Cruz et al. 2005]. Additionally, blood was also taken on the 21st day after mating in order to pregnancy diagnosis. Goats were mated with the same three fertile males.

The effectiveness of the applied programs was evaluated by the oestrus signs observation. The characteristic signs as tail wagging, bleating, swelling of the vulva, mucus discharge and actively seeking a male were observed. Also three fertile bucks were released among the females for oestrous detection and hand-mating [Zarkawi et al. 1999].

Obtained results were statistically analysed according to Kruskal-Wallis test with significant level $p < 0,01$.

## RESULTS

During the experiment, a statistically significant difference in effectiveness of the oestrus induction was found between the particular groups. Oestrus was registered in 18 out of 25 goats in group A, in group B in 6 out of 10 animals, and in group C in 10 out of 10 animals. Oestrus symptoms were not observed in any female of group D, E and F, while complete symptoms of oestrus were noted in 2 females of the control group (G). All animals with the oestrus signs were mated.

The examination of the progesterone level in the goats´ serum performed at 21 day post mating revelled its´ elevated level in 15 females of group A, 4 of group B, 7 of group C and 2 of group F. All goats with a diagnosed pregnancy in this way delivered kids in the expected time. The conception rate (percentage of females kidding / females mated) observed in particular groups was 83, 60, 70, and 100% in groups A, B,C and G respectively. Significantly higher number of the kids in the litters in group A, B and C compared to group G has been found (Tab. 1).

Table 1. The effectiveness of the estrus induction, conception rate and average fecundity in particular experimental groups

Tabela 1. Skuteczność wywołania rui, zapładnialność oraz średnia plenność w poszczególnych grupach doświadczalnych

| Groups – Grupy<br>Parameter – Parametr | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| Oestrus* – Ruja (%) | 72 | 60 | 100 | 0 | 0 | 0 | 20 |
| Conception rate** Zapładnialnośc | 83 | 60 | 80 | 0 | 0 | 0 | 100 |
| Average Fecundity*** Średnia plenność | 3,58 | 3,88 | 3,6 | – | – | – | 2 |

* percentage of the successfully oestrus induced
procent skutecznie wywołanych rui
** percentage of females kidding / females mated
odsetek samic ciężarnych do krytych
*** average number of kids per litter
średnia ilość koźląt w miocie

## DISCUSSION

In all animals from group B, C and D a significantly elevated level of progesterone was observed. According the results of researches made by Motlomelo et al. [2002] the curve representing the manner of hormone release from intravaginal sponges (CIDR) featured the initial peak and followed by gradual drop of progesterone concentration. Such a curve may seem to be advantageous while it imitates the natural growth, peak and drop of secretory activity of corpus luteum. Freitas et al. [1994] who investigated the influence of inadequate level of progestagen on the day of sponge removal on the variability in the occurrence of oestrus after hormonal treatment in anoestrus in goats, concluded that the high level of progestagens at the end of treatment could led to some loss of fertility. However, in our study obtained results showed, that the remaining through the all time of treatment a high level of progesterone, did not negatively influenced the effectiveness of the proposed program, while the best results were obtained in the group C. Progesterone administration by injection assures its stable level and allows to precisely foresee a drop of its concentration, which makes easier to fix the best time for PMSG administration.

Contrary to the parenteral administration of gestagens, an oral administration did not give the satisfactory results. A similar results was reported by Worlds et al. [2003] and Jackson and Whitley [2002], while Lerma and Stanko [2003] reported the effectiveness of the program based on the oral administration of MGA (melengestrol acetate). Analysing their own negative results as well as similar problems encountered by another researchers administrated progetagens per os Whitley and Jackson [2004] suggested the genetic background as well as local climatic conditions as a probable reasons for the oestrus induction programs failure. According to aforementioned authors, the impact of the climate, might have caused the same protocol ineffective, which had been successfully applied by other researchers [Whitley and Jackson 2004].

Considering the probable reasons for the failure in both experimental groups (E, F) it should be taken into account the difficulty in the precise administering of the drug or the poor absorption of altrenogest in goats. Problems with oral drug administration might have had an influence on gestagen level fluctuations and then on setting a wrong time of PMSG administration. The fact of oestrus occurrence in females of the control group and no oestrus detected in females of group E and F might suggest a gestagens´ blockade, which prevented the occurrence of spontaneous oestrus usually stimulated by the presence of other females in oestrus and an adult male goat.

The fact of oestrus detection in two animals from control group G, in which no protocol of oestrus stimulation was applied, may be explained by the influence of the presence of other females in oestrus. This phenomenon of the mutual sexual cycle synchronization was described in many species including humans [McClintock 1999]. Observations made during the experiment are confirmed in theses presented also by other authors [Zarco et al. 1995].

In a majority of research related to the protocols of oestrus stimulation in anoestrus goats, where different kind of progestagen (CIDR-controlled internal drug-releasing device, FGA – flugestone acetate, MAP – methyl acetoxy progesterone) were used, a similar results were obtained, while choice of the dose and the duration of treatment seems to be a still an actual problem [Greyling and Nest 2000, Holtz 2005, Leboeuf et al. 2003, Motlomelo et al. 2002, Wheaton et al. 1993]. Corteel et al. [1988] suggested that the oestrus stimulation programs in anoestrus goats needs a long-term (18–21 days) application of progestagens, which corresponded with the time of corpus luteum phase (16–18 days). However the same authors observed, that during the long lasting progestagen treatment, a significant differences in the concentration of the hormone in the blood, in particular animals could be observed. This in turn could influenced the successful rate in fertile oestrus stimulation [Corteel et al. 1988]. Analyzing the trends in modification of the programs of oestrus stimulation in out of season goats, the reduction of the exposure for the progestagen could be observed. The better results of stimulation along with increased fertility supported these modifications [Fonseca et al. 2005, Vinoles et al. 2001].

Based on the results obtained in group B, C and D we concluded that the duration of progesterone treatment had an influence on the effectiveness of oestrus induction outside of the breeding season. In case of intramuscular administration of progesterone significantly better results were obtained in the 12-day program. However, Lopez-Sebastian et al. [2007] reported the attempts of the use even single doses of progesterone in combination with the "male goat effect" and PGF2α injection (which was a reason for shortening the progesterone exposure time in one of experimental- D), the results of our study do not confirm the effectiveness of such a short time of progesterone treatment.

The higher number of kids in the litters of the females from group A, B and C comparing to group G could be explained by the influence of the PMSG (Folligon, Intervet) used in protocol of oestrus induction.

Taking into account the lack of the commercially available progestagen containing intravaginal sponges in our country, the program based on the 12 days progesterone i.m. administration, is worth recommendation, giving satisfactory results even its much work consuming.

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## PORÓWNANIE SKUTECZNOŚCI METOD WYWOŁYWANIA PŁODNEJ RUI U KÓZ POZA SEZONEM ROZRODCZYM

**Streszczenie.** Celem pracy było porównanie skuteczności różnych protokołów indukcji rui u kóz poza sezonem rozrodczym. Samice zostały podzielone na siedem grup (A–G). Zwierzętom z grupy A (n=25) zaaplikowano gąbki dopochwowe zawierające 30 mg octanu flugestonu FGA (Chronogest, Internet) na okres 12 dni oraz 500 I.U. PMSG (Foligon, Intervet) w postaci iniekcji domięśniowej na dwa dni przed usunięciem gąbek. Samice z grup B (n=10), C (n=10) oraz D (n=10) otrzymywały domięśniowe iniekcje 12,5 mg progesteronu (Progesteronum, Polfa Warszawa S.A.) odpowiednio przez okres 6, 12 oraz 2 dni. Kozy z grupy E (n=10) oraz F (n=10) otrzymywały *per os* altrenogest (Regumate, Intervet) przez okres 6 lub 12 dni. Ostatniego dnia administracji progesteronu/progestagenu samicom z grup B, C, D, E, F podano 500 I.U. PMSG i.m. Grupę kontrolną stanowiły samice z grupy G (n=10), u których nie stosowano żadnych środków hormonalnych. W toku eksperymentu stwierdziliśmy istotną różnicę w skuteczności indukcji rui u kóz pomiędzy poszczególnymi programami. Pełnowartościowa ruja wystąpiła u 72% samic z grupy A, 60% z grupy B, 100% z grupy C oraz 20% z grupy G. W grupach D, E, oraz F nie odnotowano ani jednego przypadku rui. Uzyskane wyniki wskazują na istotność długości stosowania programu opartego na podawaniu progesteronu na skuteczność wywoływania rui u kóz poza sezonem rozrodczym.

**Słowa kluczowe:** kozy, anoestrus, indukcja rui, progesteron

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