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ORIGINAL PAPER

The evolution of metallothionein (ZnMT) levels and haematological parameters in clinical and subclinical forms of copper deficiency in dairy cattle*

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Abstract

Metallothioneins are cysteine-containing proteins involved in haemostasis and the transport of zinc and copper in the body. The aim of this study was to determine the effects of copper, anaemia and metallothioneins on the severity of clinical signs in clinical, subclinical, and atypical forms of copper deficiency. The study also attempted to determine normal metallothionein reference values for dairy cattle. The study has been carried out on 67 Holstein-Friesian cows; the animals were separated into 6 groups. Some animals showed typical clinical signs indicative of copper deficiency (two groups of study animals) and low Cu concentrations in the blood. In one group of cows, there were atypical clinical signs, and the average Cu concentration was 11.15 μmol l^{-1}. In 3 study groups, no clinical signs were observed, while the average copper concentration was equal to: 14.23 μmol l^{-1}, 13.30 μmol l^{-1} and 9.87 μmol l^{-1}. Blood haematology test in all animals showed normocytic hypochromic anaemia. The authors noted that in the group with a decrease in the concentration of Cu in the blood and a high ZnMetallothionein (ZnMT), no clinical symptoms were observed in the animals, not even those related to anaemia. Clinical signs were also found to be less pronounced in cows with the highest serum Cu deficiency and significantly higher ZnMT content than in the other groups of cows with clinical signs. The conducted observations of the examined cattle in relation to Cu deficiencies, anaemia and MT content in the blood are very promising.

Keywords: cattle, metallothioneins, copper deficiency, anaemia

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INTRODUCTION

Metallothioneins (MT) are intracellular, low-molecular-weight proteins that are rich in cysteine. In the available literature (López-Alonso et al. 2005, Sakulsak 2012, Chen et al. 2020), several hypotheses concerning the functional significance of MT in mammals may be found. These proteins have the function of homeostasis and the transport of physiologically essential metals (zinc – Zn, copper – Cu); they are involved in the detoxification of metals (cadmium, mercury), protect against oxidative stress, maintain an intracellular redox balance and regulate cell proliferation and apoptosis. The rapid changes in metallothionein concentrations in response to the administration of metal-based supplements suggest that it has a regulatory function, the nature of which is not yet fully understood. MTs can bind to metal elements and reduce the content of intracellular free metals, and therefore play a major role in the detoxification of heavy metals. Tissue metallothionein concentrations depend on the body's level of Zn and, to a lesser extent, Cu, which is consistent with the theory of the induction of protein synthesis by these metals. This protein is involved in the regulation of the levels of microelements, such as zinc and copper, in mammalian cells. One of the regulatory functions of MT is to act as a zinc buffer to prevent the excessive intracellular accumulation of free Zn ions. This is accomplished through a mechanism involving Zn being transiently stored before it is incorporated into metalloenzymes, and used to support the growth and development of the body, or excreted. In turn, there is more variation in the binding of hepatic copper to metallothionein, which is dependent on multiple factors, such as the species, the route of Cu administration, and the current Zn status in the body. In most animal species, no change in metallothionein concentration is observed upon a moderate increase in dietary Cu levels. The exceptions are pigs and dogs, in which case an increase in the concentration of copper in the diet results in an increased level of this protein (Bremner, Beattie 1995, López-Alonso et al. 2005, Sakulsak 2012, Krężel, Maret 2017). The protective effect of Zn against liver damage caused by excess copper has also been attributed to the more intensive binding of Cu to metallothionein. Similarly, species that are able to store large amounts of hepatic copper in the form of metallothionein are less likely to suffer from liver dysfunction caused by the excessive accumulation of Cu (Bremner, Beattie 1995).

Zinc and copper are essential trace elements in all animal species, including cattle. These microelements play an important role in many metabolic pathways, such as the regulation of transcription or protein synthesis. The improper homeostasis of Zn and Cu results in many health disorders in cattle, which include, among others, diminished appetite, reduced milk yield and reproductive problems. Over the course of Zn deficiency, desquamation and the thickening of the skin as well as depressed immunity are observed (Hilal et al. 2016, Geboliszová, Illek, 2019). The symptoms of hypocupraemia, which is frequently observed in cows, include hair discoloration (greying) around the eyes (so-called "copper glasses"), but also on the head, neck and withers, and mobility disorders, such as lameness, joint swelling and pain. In addition, inflammation in the area of the interdigital space and the coronary band may be observed in some animals (near the limbus of cloven hooves and dermatitis interdigitalis) – Mohammed et al. (2014), Menzir, Dessie (2017), Kurek et al. (2017), Joerling, Doll (2019), Perdrizet et al. (2020). Anaemia, which is an effect of copper deficiency, is clinically manifested by the pallor of the mucous membranes, bodily weakness, accelerated breathing and the accentuation of heart sounds. Based on laboratory tests, one may observe a decrease in red blood cell parameters, such as the erythrocyte count, haemoglobin concentration, haematocrit and the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and the mean corpuscular haemoglobin concentration (MCHC). The mechanism of anaemia which occurs over the course of copper deficiency has not yet been elucidated. However, it is known that copper is necessary to maintain iron (Fe) homeostasis, as a component of proteins that are involved in haematopoiesis (ceruloplasmin and hephaestin). In addition, this trace element is utilised during the process of the production and maturation of erythrocytes in the bone marrow, and in haem synthesis through the induction of ferrochelatase activity (Collins et al. 2010, Roland et al. 2014, Hagmeyer et al. 2015, Kurek et al. 2017, Chen et al. 2020).

The aim of the study was to determine the effect of copper, anaemia and metallothioneins on the severity of clinical symptoms in clinical, subclinical and atypical forms of copper deficiency. In this study, an attempt was also made to determine normal metallothionein reference values for dairy cattle.

MATERIALS AND METHODS

Animals

The study was carried out on 67 Holstein-Friesian cows, aged 3 to 6 years, at the peak of lactation, with an average BCS of 3.25/5 points, which came from two indoor dairy farms in Silesia, Poland. The milk production of these cows was found to be in the range of 9,000-10,000 kg of milk, which was determined previous to the study. Nutrition was based on the TMR method, and the ration included: maize silage, ground and whole maize grain, haylage, grass silage, hay, straw, granulates, soybean meal, on-farm-grown cereals, feed additives with a protein content of 18-24%, premixes and mineral and vitamin supplements. The feed ration was designed on the basis of milk yield, as well as the physiological period and body weight of the cows. The animals were under regular veterinary care. In these herds, hoof trimming was performed twice a year and prophylactic deworming was also carried out (spot-on delivery method, eprinomectin). In order to qualify for the study, only those animals free from any parasitic and infectious diseases, injuries and bleeding (from the skin, digestive or urinary tract) were included. Animals that had experienced complications at parturition were also rejected from the study. On the basis of the conducted clinical examination and various laboratory tests, the animals which qualified for the study were divided into six groups (Tables 1 and 2). The first group consisted of cows with clinical symptoms, such as so-called "copper glasses", the greying of the coat on the head, neck and forelegs, a reduction in milk yield, and in some animals (over 30%) various degrees of lameness and dermatitis interdigitalis. The average serum Cu concentration was 8.96 μmol l^{-1}. The second group included animals with less pronounced clinical symptoms, most often in the form of "copper glasses" and a lower milk yield, as well as varying degrees of lightening of the hair in the head and neck area. The third group consisted of animals with a slight decrease in milk production, as well as lameness caused by inflammation in the cloven hooves (coronary band and dermatitis interdigitalis). These animals were included after the determination of the copper concentration (average concentration 11.15 μmol l^{-1}) and the attainment of a positive effect through supplementation with copper licks. The fourth group consisted of clinically healthy animals with normal blood copper levels and low ZnMT levels. The fifth group consisted of clinically healthy animals with normal copper concentrations and high ZnMT concentrations in the blood. The sixth group included animals without clinical symptoms but with significant copper deficiency (average concentration of 9.87 μmol l^{-1}) in the blood. Copper licks and mineral supplements containing an increased amount of Cu were administered to all animals with reduced serum copper concentrations (studies in progress).

Research material

Before blood sample collection, the health status of the cows was assessed by interviewing the livestock keeper; this was followed by the clinical examination of the animals; in addition, the information was collated concerning the feed administration method, the quality of the feed and milk yield. Blood was collected in the morning during or after milking and before the first feeding. The blood was collected from the external jugular vein into tubes containing K_3EDTA and those with a clotting activator. The clot activator tubes were centrifuged at 3,000 revolutions min^{-1} for 7 min to obtain serum.

Methods

For the blood hematology test, the red blood cell count (RBC), haemoglobin concentration (Hgb), haematocrit (Hct), MCV, MCH and MCHC and leukocyte (WBC) and platelet (PLT) levels were determined using an Horiba scil Vet abc Plus automatic analyser and reagents dedicated to the instru-

Table 1

Haematological values means

Parameters	Bovine reference intervals according to Baumgartner (2005)		Groups					
			I *N*=14	II *N*=13	III *N*=12	IV *N*=8	V *N*=9	VI *N*=11
RBC $x10^{12}$ l^{-1}	5-7	X±SD median	6.28±0.81 6.28	6.55±0.65 6.85	7.19±1.12 7.27	6.60±0.70 6.37	6.31±0.80 6.22	6.32±0.19 6.29
Hb (gl^{-1})	105-140	X±SD median	77.33±26.70 86.00	92.27±10.12 95.00	98.00±13.79 100.50	87.38±6.80 85.50	92.00±8.32 94.00	93.00±5.18 90.50
Hct (l l^{-1})	0.30-0.40	X±SD median	0.26±0.03 0.27	0.30±0.03 0.31	0.32±0.05 0.33	0.28±0.02 0.28	0.29±0.3 0.28	0.29±0.02 0.29
MCV (fl)	40-60	X±SD median	44.11±3.18 45.00	45.45±3.67 46.00	44.38±5.58 44.00	42.50±4.04 42.50	42.17±3.92 43.00	45.00±2.53 45.00
MCH (fmol)	0.9-1.5	X±SD median	0.79±0.07 0.77	0.85±0.11 0.84	0.88±0.07 0.86	0.83±0.08 0.82	0.86±0.06 0.88	0.88±0.04 0.87
MCHC (mmol l^{-1})	16-21	X±SD median	19.45±0.41 19.41	19.31±0.28 19.34	19.30±0.25 19.28	19.44±0.43 19.47	19.43±0.23 19.44	19.53±0.25 19.56
WBC $x10^{9}$ l^{-1}	6.2-9.5	X±SD median	8.07±2.00 7.90	7.89±1.43 7.30	8.03±0.96 7.90	8.04±2.66 7.15	9.32±2.86 9.45	8.30±2.94 7.45
PLT $x10^{9}$ l^{-1}	200-800	X±SD median	739.22±206.98 717.00	603.55±128.55 633.00	534.00±105.12 543.00	601.62±370.06 635.50	794.00±180.33 734.50	748.33±222.71 799.00

Table 2

Results of biochemical tests in cows

Parameters	Bovine reference intervals according to Dirksen (2002)		Groups					
			I *N*=14	II *N*=13	III *N*=12	IV *N*=8	V *N*=9	VI *N*=11
Fe (µmol l^{-1})	21.5-38.5	X±SD median	29.63±10.88 26.11	33.71±11.08 33.74	29.38±9.61 29.61	29.72±13.06 30.68	25.71±14.39 24.39	29.53±8.17 29.01
Cu (µmol l^{-1})	12-20	X±SD median	8.96±0.66 8.69	6.55±0.59 6.99	11.15±0.52 11.29	14.23±1.64 14.69	13.30±2.80 12.71	9.87±1.32 9.78
Zn (µmol l^{-1})	7.7–22.9	X±SD median	11.73±4.95 10.64	11.74±2.88 11.23	11.96±3.81 10.14	11.30±1.78 9.36	11.81±2.90 11.15	11.32±3.18 12.16
ZnMT		X±SD median	23.05±8.66 24.25	48.20±9.87 34.57	25.33±12.72 21.55	23.87±7.94 25.58	119.04±30.20 115.51	117.50±36.18 108.40

ment. Cu, Fe and Zn were quantified using atomic absorption spectrometry (AAS) with sample atomization based on an acetylene-air flame. The concentration of Zn metallothionein was determined using commercially available ELISA tests – Bovine Zn Metallothionein (ZnMT) and an ELISA kit (BlueGene) by following the procedures recommended by the manufacturer.

The results obtained in the study were subjected to mathematical statistical analysis by means of a t-Student test using Statistica® 10.0 software (Statsoft).

RESULTS AND DISCUSSION

The study covered cattle herds which were assumed to be affected by clinical and subclinical copper deficiency. In these herds, there were observed symptoms indicating a deficiency of this element to a varying degree of severity – from greying of the coat around the eyes, to inflammation in the dermatitis interdigitalis and a decrease in the milk yield. The division into experimental groups did not allow for an explanation to be found elucidating the differences between the groups or explaining the absence of symptoms in the group with a significant decrease in Cu concentration (group VI). It was also problematic to explain the different degrees of anaemia in the animals studied. In the present study, different levels of ZnMT in the blood were detected, and this parameter was used as a criterion for the division of the animals into individual groups. This protein attracted the attention of the authors due to its important role in the transformation of microelements, mainly copper and zinc. There is evidence that this protein is involved in the cellular detoxification of Cu, and that it has been involved in the accumulation of Cu bound to metallothionein in the liver (Bremner, Beattie 1995, López-Alonso et al. 2005, Chen et al. 2020). According to the knowledge of the authors of this article, no studies presenting the relationship between copper deficiency, anaemia and the levels of blood metallothioneins in cattle have been published to date.

Group I had the most pronounced symptoms of copper deficiency, which was later confirmed by the results of blood concentration of this element – 8.96 μmol l^{-1} (Table 2). These values were statistically lower than those obtained in groups III, IV, V and VI, but at the same time significantly higher than in group II. Animals in this group were characterized by pallor of mucous membranes, accelerated breathing and accentuation of heart sounds. This was due to the occurrence of anaemia, which was confirmed by a haematological test. In cows from this group, the haemoglobin concentration was the lowest out of all the examined groups and a decrease in the Hct and MCH was found, which indicates the development of normocytic hypochromic anaemia. In the animals of the first group, the blood iron levels were within the accepted norms (Dirksen 2002). Similar observations were made in studies of humans and cattle, where the same type of anaemia was observed in the state of copper deficiency (Lazarchick 2012, Abramowicz et al. 2021). To date, in the context of copper deficiency in animals, the development of the microcytic, hypochromic anaemia has been mostly reported (Mohammmed et al. 2014, Roland et al. 2014). From the analysis of the obtained blood test results, it emerged that the concentration of ZnMT in the blood in this group was the lowest of all the examined groups and significantly lower compared to groups II, V and VI.

In group II, lesser severity of clinical symptoms was noted, although they were still characteristic of copper deficiency (Table 3). In the biochemical blood test, the lowest average Cu concentration was noted – 6.55 μmol l^{-1}, statistically significantly lower than in the other groups. On haematological blood test, a decrease in haemoglobin concentration and MCH was observed, other red blood cell parameters were within the accepted standards (Baumgartner 2005) – Table 1. These changes might have occurred due to low concentration of Cu in the blood of the tested animals. Collins et al. (2010) observed that during copper deficiency in the body, the production of Hgb is ineffective, despite the normal concentration of iron in the blood. The mechanism of anaemia development over the course of Cu deficiency is still not fully clarified, although it may be linked to copper acting as an essential coenzyme involved in the oxidation of Fe and its transport in the body (Collins et al. 2010, Luo et al. 2021). In this group, ZnMT was significantly higher than in groups I, III, IV and significantly lower than in groups V and VI (Table 2).

In group III, a slightly reduced concentration of Cu in the blood was found, which indicates a chronic, long-term subclinical deficiency of this element. Copper concentration values in this group were significantly higher than in groups I and II and significantly lower than in group IV. Animals included in this group showed nonspecific clinical symptoms (Table 3). In herds where the status of copper is unknown, such symptoms may be attributed to many diseases. On the other hand, in herds with hypocupraemia, the animals often display an atypical or subclinical course of deficiency disease. In haematological blood test, as in group II, a decrease in Hgb concentration and MCH was observed (Table 1). These parameters were higher than in group II, and the concentration of Cu in this group was almost twice as high. Based on the analysis of own results, it was found that changes in Hgb and MCH indices result from long-term copper deficiency. The literature data (Perdrizet et al. 2020) and own observations may lead to a conclusion that the deficiency of this element is difficult to diagnose because the concentration of Cu in the blood remains at a constant level until the copper stored in the liver is exhausted. The content of ZnMT in this group was very similar to the level in groups I and IV, but significantly lower compared to groups II, V and VI (Table 2).

Due to the lack of clinical symptoms, at the initial stage of the study cows from groups IV, V and VI were classified into one group as healthy an-

Table 3

Clinical symptoms in animals

Groups	Clinical symptoms
I	- so-called "copper" glasses - greying of the coat of the head, neck and forelegs - reduction in milk yield - various degrees of lameness and inflammation in the interdigital space – in more than 30% of animals - pallor of the mucous membranes, - tachypnoea - accentuation of heart sounds
II	- so-called "copper" glasses - reduction in milk yield - varying degrees of lightening of the hair coat around the head and neck
III	- a slight reduction in milk yield - lameness caused by inflammation within the cloven hooves (coronary band and dermatitis interdigitalis)
IV	no clinical symptoms
V	no clinical symptoms
VI	no clinical symptoms

imals. After receiving the results of morphological and biochemical blood tests, they were divided into 3 additional groups. A reduction in Hgb concentration, Hct value and MCH was observed in these groups (Table 1). These parameters did not show statistically significant differences in individual groups. In group IV, the highest concentration of Cu among all examined groups and a very low concentration of ZnMT were found. In group V, the Cu content was clearly lower, but within the accepted standards (Dirksen 2002). In this group, the highest content of ZnMT was also observed. In group VI, despite the lack of symptoms, a low average concentration of Cu in the blood was found (9.87 μmol l^{-1}), which was significantly lower than in group III, in which clinical symptoms occurred. Diminished red blood cell parameters (Hgb, Hct and MCH) were higher than in groups IV and V. At the same time, a high content of MT was found in this group, statistically significantly higher than in groups I, II, III, IV (Table 2).

The authors noted that in the group with a significant decrease in the concentration of Cu in the blood and a high ZnMT (group VI), no clinical symptoms were observed in the animals, not even those related to anaemia. From own observations and literature data (Roland et al. 2014, Menzir, Dessie 2017), it has been demonstrated that the concentration of Cu in the range of 9.87 μmol l^{-1} in the serum is often accompanied by dyspnoea, tachypnoea and accentuation of heart sounds. The only clinical sign common to all copper-deficient cows studied was a mucosa membranes that were paler than in those not suffering from deficiency. Clinical symptoms were also less pronounced in cows with the lowest serum Cu levels, and, at the same time, a ZnMT content which is significantly higher than in other groups of cows with clinical symptoms, was detected.

The overall results suggest that MTs are the proteins that are not only involved in the detoxification of Cu in the liver, but also affect the storage or transport of Cu in the blood in the cases of deficiency (López-Alonso et al. 2005, Sakulsak 2012). Own observations of the examined cattle in relation to Cu deficiencies, anaemia and MT content in the blood are very promising. However, the interpretation of the presented results is difficult because the noted changes have not yet been explained and require further research. The available literature does not specify the standard MT content in the blood of cattle. Our studies also failed to clearly define the correct MT values in cattle. However, a clear relationship was observed between the occurrence of clinical symptoms of copper deficiency and the concentration of MT. In the authors' research, a higher concentration of ZnMT was always associated with a milder course or a lack of clinical symptoms of Cu deficiency (groups II and VI). It may be concluded that the animals that have low MT levels and hypocupraemia should be treated first and with higher doses of Cu. This conclusion is based on the authors' own research, which will be published after the completion of the overall study, in which the administration of Cu licks and mineral supplements containing an increased amount of Cu in all deficient groups brought the most beneficial effects in animals with high or very high MT levels.

REFERENCES

Abramowicz B., Kurek Ł., Chałabis-Mazurek A., Lutnicki K. 2021. *Copper and iron deficiency in dairy cattle*. J. Elem., 26(1): 241-248. DOI: 10.5601/jelem.2020.25.4.2093

Baumgartner W. 2005. *Clinical propaedeutic of internal diseases and skin diseases of domestic animals*. 6th ed., Parey, Berlin, Germany, pp. 207-226. (in German)

Bremner I., Beattie J.H. 1995. *Copper and zinc metabolism in health and disease: speciation and interactions*. Proc. Nutr. Soc., 54(2): 489-99. DOI: 10.1079/pns19950017.

Chen G.H., Lv W., Xu Y.H., Wei X.L., Xu Y.C., Luo Z. 2020. *Functional analysis of MTF-1 and MT promoters and their transcriptional response to zinc (Zn) and copper (Cu) in yellow catfish Pelteobagrus fulvidraco*. Chemosphere, 246,125792. https://doi.org/10.1016/j.chemosphere.2019.125792

Collins J. F., Prohaska J.R, Knutson M.D. 2010. *Metabolic crossroads of iron and copper*. Nutr. Rev., 68(3): 133-47. DOI: 10.1111/j.1753-4887.2010.00271.x

Dirksen G. 2002. *Diseases of the liver and gallbladder*. In: *Internal Medicine of Cows*. Dirksen G., Gründer H.D., Stöber M. 4th ed. Parey im Blackwell, Berlin, Germany, pp. 649-664. (in German)

Geboliszová K., Illek J. 2019. *Zinc and its function in cattle*. Veterinářství, 69(6): 368-370.

Hagmeyer S., Haderspeck J.C., Grabrucker A.M. 2015. *Behavioral impairments in animal models for zinc deficiency*. Front Behav. Neurosci., 6(8): 443. DOI: 10.3389/fnbeh.2014.00443

Hilal E.Y., Elkhairey M.A.E., Osman A.O.A. 2016. *The role of zinc, manganese and copper in rumen metabolism and immune function: A review article*. Open J. Anim. Sci., 6: 304-324. http://dx.doi.org/10.4236/ojas.2016.64035

Joerling J., Doll K. 2019. *Monitoring of iron deficiency in calves by determination of serum ferritin in comparison with serum iron: A preliminary study*. Open Vet. J., 9(2): 177-184. DOI: http://dx.doi.org/10.4314/ovj.v9i2.14

Krężel A., Maret W. 2017. *The functions of metamorphic metallothioneins in zinc and copper metabolism*. Int. J. Mol. Sci., 9; 18(6): 1237. DOI: 10.3390/ijms18061237

Kurek Ł., Olech M., Lutnicki K., Riha T., Brodzki P., Gołyński M., Abramowicz B. 2017. *Long-term subclinical copper deficiency and it is influence on functions of parenchymal organs and the serum macro-element deficiency in dairy cows*. J. Elem., 22(4): 1415-1425. DOI: 10.5601/jelem.2017.22.1.1417

Lazarchick J. 2012. *Update on anemia and neutropenia in copper deficiency*. Curr. Opin. Hematol., 19: 58-60. DOI: 10.1097/MOH.0b013e32834da9d2.

López-Alonso M., Prieto F., Miranda M., Castillo C., Hernández J., Benedito J.L. 2005. *The role of metallothionein and zinc in hepatic copper accumulation in cattle*. Vet. J., 169(2): 262-267. DOI: 10.1016/j.tvjl.2004.01.019

Luo T., Zurko J., Astle J., Shah N.N. 2021. *Mimicking myelodysplastic syndrome: Importance of differential diagnosis*. Case Rep. Hematol., 29: 9661765, https://doi.org/10.1155/2021/9661765

Menzir A., Dessie D. 2017. *Review on copper deficiency in domestic ruminants*. IJARP, 1: 101-110.

Mohammed A., Campbell M., Youssef F.G. 2014. *Serum copper and haematological values of sheep of different physiological stages in the dry and wet seasons of central Trinidad*. Vet. Med. Int. DOI: 10.1155/2014/972074

Perdrizet U., Blakley B., Al Dissi A. 2020. *Concentrations and deficiencies of minerals in cattle submitted to a diagnostic laboratory in Saskatchewan from 2003–2012: A retrospective study*. Can. Vet. J., 61: 57-62.

Roland L., Drillich M., Iwersen M. 2014. *Hematology as a diagnostic tool in bovine medicine*. JVDI, 26: 592-598. DOI: 10.1177/1040638714546490

Sakulsak N. 2012. *Metallothionein: an overview on its metal homeostatic regulation in mammals*. Int. J. Morphol., 30(3): 1007-1012.