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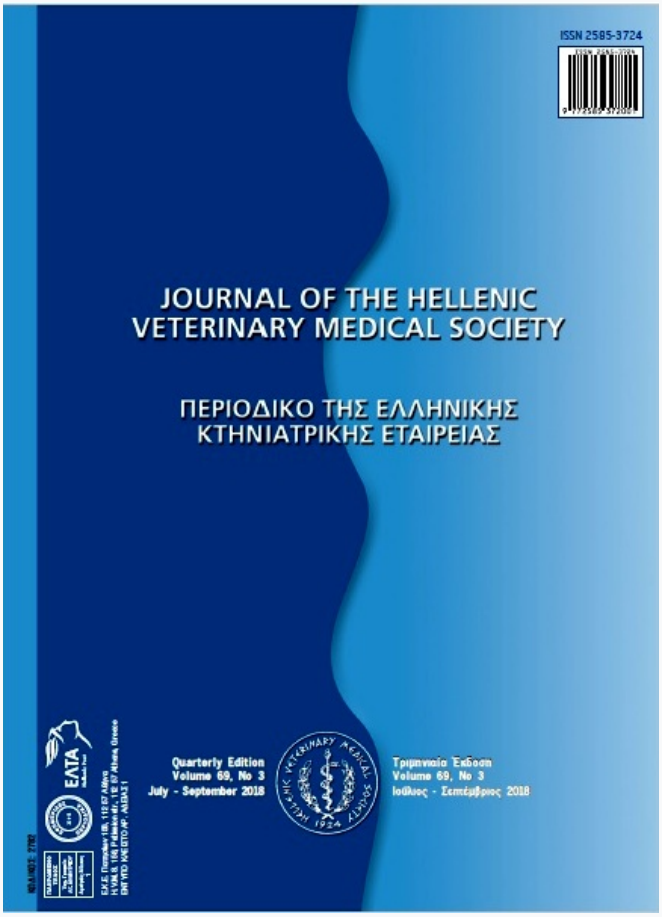

Stability of HCT, HGB and RBC values in the Mute Swan (Cygnus olor) blood stored at 4°c and 24°c differs between traditional and multi-parameter automated methods

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Short communication
Σύντομη δημοσίευση

Stability of HCT, HGB and RBC values in the Mute Swan *(Cygnus olor)* blood stored at 4°c and 24°c differs between traditional and multi-parameter automated methods

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ABSTRACT. The objective of this study was to evaluate the influence of time of sample storage, method of analysis, and storage temperature on stability of HCT, HGB, and RBC in avian blood samples. Blood samples from mute swans were stored at 24°C or 4°C. Analyses of HCT, HGB, and RBC were carried out after 5 and 25 hours after collection of blood using both traditional and Cell-Dyn 3700 analyzer methods. Storage temperature had no significant influence on HCT, HGB, and RBC values of mute swan blood. However hematological parameters obtained by the traditional method were significantly lower than the results from the analyzer. Time of storage had a significant influence on values obtained traditionally for HCT, HGB, and RBC, but did not affect results that were obtained by the multi-parameter automated method. These results indicate that the mute swan blood can be stored at both room and refrigerator temperature. However, the analysis should be performed as soon as possible, especially in the case of traditional methods. Reference intervals for this species should always include information about the method used for hemogram determination and time elapsed since the blood collection.

Keywords: Avian blood, Cell-Dyn 3700 analyzer, Mute Swan, storage time, temperature.

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INTRODUCTION

The key parameters included in the hemogramare: packed cell volume (HCT), hemoglobin (HGB), and red blood cell count (RBC) (Ihedioha et al. 2007; Owen 2011). It is known that the number of preanalytical factors, such astime of storage before analysis, method of analysis, and storage temperature of the sample, may influence the final value of hemogram parameters (Hadzimusic et al. 2010). Although it has been suggested that avian blood should be stored in a refrigerator before the analysis (Hadzimusic et al. 2010; Owen 2011), there have been no reports of studies on artifactual changes in avian hematological parameters determined either by traditional (manual) ormulti-parameter automated analysis. This study evaluated the influence of storage time, storage temperature, and analysis method on the variation in RBC, HCT, and HGB values in avian blood.

The model bird species chosen for our study was the mute swan *Cygnus olor*. This species is often treated in wildlife rehabilitation centers, both in Europe and in North America, where diagnostic CBC tests are performed in order to evaluate their condition status, especially before releasing them into the wild (e.g. The Swan Sanctuary: http:// www.theswansanctuary.org.uk, Wings Wildlife Rehabilitation Centre: http:// www.wingsrehab.ca). Mute swans weighing 4–16 kg are a good model for this CBC study, as it is possible to obtain large quantities of blood from one individual, which is necessary to carry out different hematological analyses.

MATERIALS AND METHODS

Sample collection

Blood samples were obtained from 10 adult males and 10 adult females of mute swans wintering along the municipal Baltic coast of Gdańsk, Sopot, and Gdynia on the Polish Baltic coast. Body masses of birds used in this study were in the range of those given in the literature for the non-breeding period (7.47–11.73 kg in females, and 8.06–12.03 kg for males) (Wieloch et al. 2004).Thus, it was assumed that blood samples were obtained from mute swans that were in good condition. Within 10 minutes of the swans beingcaught, blood (2 ml) was collected from the metatarsal vein into a test tube containing EDTA anticoagulant. Capture of birds and blood collection were allowed based on a Permit of the Local Ethics Committee (no. 61/2012). Blood samples were collected in the morning hours from no more than four birds at a time. The exact time of blood collection from a particular individual was recorded. All samples were then transported to the laboratory (time of transit ranged from 30 to 40 minutes). All blood samples were divided into two aliquots: one was intended for traditional analyses, while the other was used for multi-parameter automated analysis.

Laboratory analysis

Both traditionally and automatically analyzed samples were then split further into twoequal parts: one part was kept on the work-topin the laboratory at room temperature (~24°C), while the other part was stored in a refrigerator (4°C).Hematologic analyses were carried out for all samples at 5 and 25 hours after collection of blood using both traditional and multi-parameter automated methods.

The only parameter determined for ambient temperature only, and not for refrigerator temperature,was traditional HCT obtained 3 hours after blood collection. This exception was due to the fact that a pilot study showed that at~4 hours after collection, swan blood very often becomes hemolysis in the microhematocrit centrifuge and hence, these results become unreliable. After 4 hours from collection of blood, samples stored at 4°C and those at 24°C did not differ and 80% of them had no signs of hemolysis. After 5 hours, only 15% (samples stored at 24°C) and 5% (samples stored at 4°C) had no signs of hemolysis. At 24°C hemolysis occurred more slowly (G test, P=0.003). After 10 hours at least a partial hemolysis was observed in all samples. 70% of the samples stored at 24°C and 80% of the samples stored at 4°C have been completely hemolysis after 10 hours (Fig. 1). However, in the case of determination made by the analyzer, such a phenomenon has not been observed.

Hematologic determinations were carried out following standard traditional laboratory procedures for avian bloodand in hematological analysis.[9] In traditional method HCT was determined by the microhematocrit method (10640 x g; Microhematocrit Centrifuge Type 346, Unipan, Warsaw 00-818, Poland).The HGB concentration was obtained using the cyanmethemoglobin method (elimination of nuclei: centrifugation

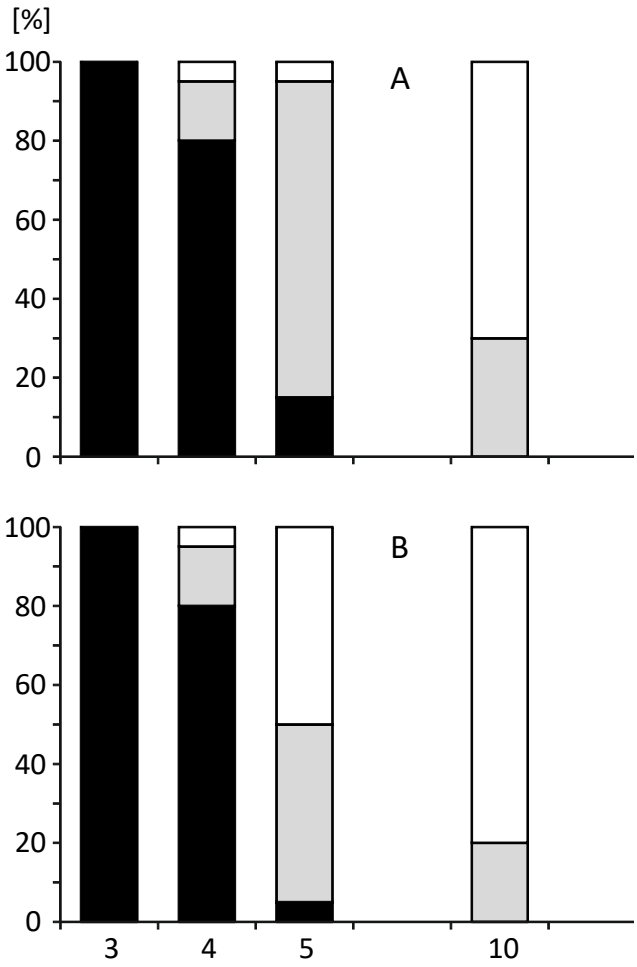

Fig 1:. Comparison of swan blood hemolysis in the microhematocrit centrifuge after: 3, 4, 5 and 10 hours from sample collection (A – samples kept at 24°C, B – samples kept at 4°C). Black bar – no signs of hemolysis, grey bar – partial hemolysis, white bar – complete hemolysis.

at 1000 x g for 10 min): Drabkin's solution (Analab, Warsaw 02-055, Poland) and spectrophotometer (7 Series Spectrophotometer Model 722, Hinotek, Ningbo, Zhejiang 315040, China) with wavelength 540 nm. RBC was determined using a hemocytometer (an improved Neubauer counting chamber). Before the count, blood was diluted 200 timeswith saline solution. Diluted blood was applied to the Neubauerchamber (Marienfeld Superior, Lauda-Königshofen, Bavaria 97922, Germany). Erythrocytes were counted in all squares exactly 3 min after application (magnification x400, microscope Ken-A-Vision, Kansas City, MO 64133, USA). To obtain the final RBC in 1μl, the sum of cells counted in all squares was multiplied by 10 (as the volume of the chamber, 0.1μl, multiplied by 10 gives 1μl) and finally the result was multiplied by the dilution (x200). Counting of every sample was repeated twice. The final result was the average of the two counts. Differences between the two results obtained for counting of the same sample did not exceed 5%.

Automated determinations were carried out using hematological analyzer Cell-Dyn 3700 (Abbott, Abbott Park, IL60064, USA) and the Veterinary Package Program was applied. Before analyses, the analyzer was configured on the basis ofpre-programmed configuration files designed for domestic (Pekin) duck (DUCK MODE). The appropriate analyzer settings were computed for a newly created file intended for swans by running blood samples from the mute swan on the analyzer. The position of each scattergram and histogram (COMPLEXITY, LOBULARITY, RBC, and PLT) were then compared to the reference scattergrams and histograms (included in the analyzer'smanual). Adjustment factors were computed from these data for electronic gain settings. Final configurations of gain settings for mute swan used in this study were as follows: WOC 0D: 2896, WOC 10D: 1500, WOC 90D: 2996, WOC 90DP: 2278, RBC: 1200, PLT: 2727, WIC: 3325.

HGB is directly measured by the Cell-Dyn 3700 analyzer using a modified hemoglobin hydroxylamine method, in which a filtered LED with a wavelength of 540 nm is the light source. A photodetector measures the light that is transmitted, and the HGB concentration is expressed in grams of hemoglobin per deciliter. RBC is counted using electrical impedance. HCT is calculated by the analyzer according to the obtained values of RBC and MCV.

Statistical analysis

Repeated measures ANOVA was used for a comparison of mean RBC and HGB values at 5 and 25 hours after bleeding. The applied method (traditional or Cell-Dyn) and temperature (room temperature and refrigerator) was used as discrete factors.In the case of HCT measurements, two different analyses were conducted. The results obtained from Cell-Dyn were compared using repeated measures ANOVA with two levels of temperature as the discrete factor. Microhematocrit results were compared only with the first reading (after 5 hours) by the Cell-Dyn by a single factor ANOVA. Assumption of sphericity was not violated in all repeated measures

ANOVA analyses (Mauchly'sphericity test, p>0.05 in all cases), andhence, univariate analysis was selected. The Tukey test was used for post-hoc comparisons after ANOVA.All calculations were conducted using Statistica 10 software (StatSoft Inc., Tulsa, OK 74104, USA). Significance level was set at p<0.05.

RESULTS

There were no statistically significant differences among mean HCT values obtained after 5 and 25 hours in analyzer (p=0.43). Temperature had no influence on mean HCT (p=0.96). Mean HCT obtained by microhematocrit method was significantly lower than both mean results from analyzer (p<0.001) (Fig. 2).

Mean HGB was significantly higher in the case of the Cell-Dyn method compared to the Drabkin's method after both 5 and 25 hours (p<0.001, Fig.3). Bythe Drabkin'smethod, there was a statistically significant decrease in mean HGB between measurements taken at 5 and at 25 hours after bleeding (p=0.04).

In the case of RBC, count method (p<0.001) and time (p<0.001) had a significant influence on the obtained results with a statistically significant interaction between these factors (p<0.001). Storage temperature had no significant effect on RBC count result (p=0.08). There were no statistically significant differences between mean Cell-Dyn counts after 5 and 25 hours (p=0.91), but mean RBC traditional counts decreased gradually with time (p<0.001), while Cell-Dyn count remained at the same level (Fig. 4). Ateach time-point,

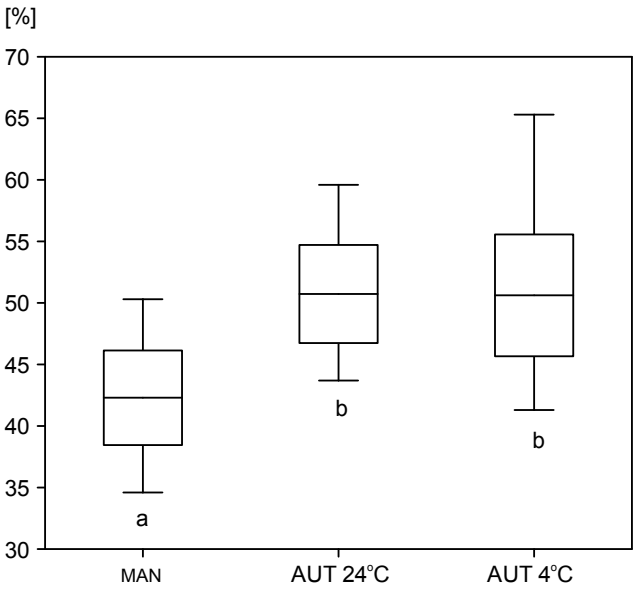

Fig 2:. Comparison of mean HCT obtained by traditional method (MAN) and by multi-parameter automated method after storing of the sample in room temperature (AUT 24°C) and in refrigerator (AUT 4°C). Horizontal line – mean, rectangle – standard deviation, vertical line – range. Letters designate means not significantly different from each other (p<0.05).

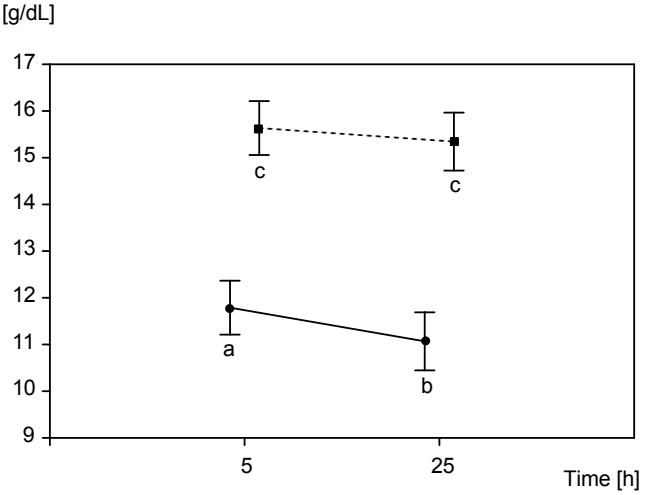

Fig 3:. Comparison of mean HGB obtained by traditional (dots, solid line) and multi-parameter automated method (squares, dashed line) after 5 and 25 h from bleeding. Vertical line shows 95% confidence limit. Letters designate means not significantly different from each other (p<0.05).

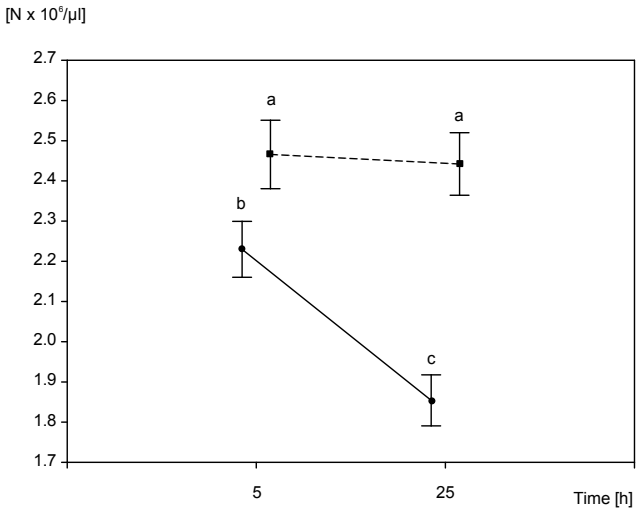

Fig 4: Mean RBC count using Cell-Dyn (dots, dashed line) and traditional method (squares, solid line) after 5 and 25 hours after bleeding. Vertical lines show 95% confidence intervals. Letters designate means not significantly different from each other (p< 0.05).

the Cell-Dyn RBC count was significantly higher than that from the traditional count $p<0.003$ in both cases).

DISCUSSION

Sample stability is the ability of its analyte to maintain the initial value of the determined parameter. Analyte stability, however, may vary due to storage temperature of the sample, time elapsed since blood collection, animal species, and method of analysis (Ameri et al. 2011).In this study, temperature of sample storage had no significant effect on analyte stability for all of the determined parameters (HCT, HGB, and RBC) regardless of the determination method (traditional or multi-parameter automated method). Based on other studies, temperature may have a different impact on sample stability in various mammalian species (Ihediohaet al. 2007). There are few data on the effect of storage temperature on the stability of avian hematological parameters. However, it was suggested that turkey's blood should be stored at refrigerator temperature before hematological analysis (Hadzimusic et al. 2010). It has also been demonstrated that storage temperature may differently affect the fragility of erythrocytes of different bird species. For example, storage of blood at 10°C for 24 hours causes a significant decrease in the fragility of pigeon *Columba livia forma domestica* and peafowl *Pavo cristatus* erythrocytes and a significant increase inthe erythrocyte fragility in domestic fowl *Gallus gallus domesticus* (Oyewale 1994). Therefore, storage temperature should be stable to provide comparable conditions for the determination of hemogram in the subsequent blood samples.

Time of storage and method of analysis had a significant influence on the stability of the mute swan HCT, HGB, and RBC. For all of the measured parameters, higher stability was observed in the case of the multi-parameter automated analysis method. Moreover, values of HCT, HGB, and RBC were higher when determined automatically. The differences between susceptibility to storage time of traditionally and automatically measured parameters are most probably due to the higher fragility of the mute swan erythrocytes in the case of traditional methods. There are numerous studies that show that the fragility of erythrocytes of different bird species varies to agreat extent (Viscor and Palomeque1982; Oyewale and Durotoye 1988; Oyewale 1992. Even within one species of birds, fragility of erythrocytes may vary significantly among different breeds, even though their hemogram parameters are identical, which was shown in three breeds of chicken (Oyewale and Durotoye1988). Interspecies differences in erythrocyte fragility with similar hemogram values suggest that red blood cells of different bird species may react differently in the same conditions. Even small differences in hemoglobin structure may result in very distinct physiological properties of erythrocytes (Oberthür et al. 1982; Habibu et al. 2013). In the case of HCT, the stability of the values obtained after traditional analysis was so susceptible to the time of storage that, after 5–6 hours since blood collection, the results obtained by the microhematocrit method were not reliable due to substantial hemolysisthat occurred in micro-capillaries. This was due to the fact that, during centrifugation of blood in micro-capillaries, the majority of the mute swan erythrocytes were destroyed duringthe longer time of blood storage, because the erythrocyte fragility increases. In the case of the multi-parameter automated method, HCT was not determined directly, but was calculated on the basis of two other parameters: MCV and RBC. Therefore, the high fragility of the mute swan erythrocytes did not affect the obtained result. These results are in agreement with other published data, where the mute swan HCT obtained traditionally was lower (21–44%) than HCT determined by the analyzer method (35–50%) (Routh and Sanderson 2010; Dolka et al. 2014).

Furthermore, RBC determined traditionally decreased significantly during the storage of samples, whereas RBC measured automatically did not change during the time of blood storage. In the case of RBC obtained by the traditional method, erythrocytes were diluted in saline solution and remained there for at least 7 minutes (mixing in saline solution, application to a Neubauer chamber, and counting). Unlike in the case of the traditional counting method, RBC values that were measured automatically were determined almost immediately after the aspiration of the blood sample. Therefore, the duration of diluent's influence on erythrocytes was much shorter (only a few seconds), and so majority of erythrocytes remained unhemolysed. This result also agrees with other data published for themute swan, where RBC values obtained by the traditional method were lower (1.72–2.43 x 10^6/μl) than values determined

by the analyzer method (1.85–2.86 x 10^6/µl) (Routh and Sanderson 2010; Habibu et al. 2013).

In the case of HGB, the significant instability of values obtained using the Drabkin's method may be connected to the interspecies differences between erythrocytes (e.g., high fragility of erythrocytes in the mute swan). Perhaps also the hemoglobin of various avian species may react differently in comparable conditions. For example, it is known, that amino acid sequence of hemoglobin's chains in the Canada goose *Branta canadensis* is very similar to the mute swan hemoglobinsequence (Oberthür et al. 1982). However, oxygen affinity of the mute swan's hemoglobin is much higher (Oberthür et al. 1982). The Drabkin's method is based on the reaction, in which hemoglobin and some of its derivatives are oxidized by $K_3[Fe(CN)_6]$ to methemoglobin, and then converted under the influence of KCN in a stable compound: cyanmethemoglobin (Drabkin 1949). It can be assumed that the hemoglobin in mute swans is in some way different from the hemoglobin of other avian species, in which the Drabkin's method was successfully used, as it is recommended for many avian species (Owen 2011). However, in the studies where the Drabkin's solution method was used to determine the mute swan HGB, the range of values was very wide. For example, in data published by Dolka et al. (2014), the range of HGB values for normal, adult mute swans was 8.5–24.6 g/dl. In comparison, the range of values obtained by other authors using multi-parameter automated methods is much narrower: 11–16.5 d/dl or 10.6–16.1 g/dl (Flinchum 2006; Dolka et al. 2014). This data are in agreement with our results, where the range of HGB determined traditionally is much wider (7.1–19.7 g/dl) than the values of HGB measured automatically (12.2–19.2 g/dl). Moreover, in our study, there was a significant difference between HGB values determined with the two different methods, even for the results obtained 5 hours after blood collection. Results of HGB that was measured automatically were not only more stable than the traditional ones, but also similar to the data published by other authors. Therefore, it is suggested that the method using Drabkin's solution and centrifugation should either not be used for the mute swan, or the results obtained by this method should only be compared with HGB values determined by the same method. Moreover, it is suggested that in the case of the mute swan hemogram reference intervals, the time of sample storage before the analysis should always be stated, at least in the case of traditional methods of determination, as previously this information has beenomitted by some authors (Dolka et al. 2014).

In conclusion, the mute swan blood can be stored both at room temperature and refrigerator temperature. However,the analysis should be performed as soon as possible, especially in the case of traditional methods. It is recommendedto determine hematological parameters of the mute swan within 3–4 hours in the case of the traditional method. It is suggested that the results obtained for the mute swan hemogram by traditional and multi-parameter automated methods should be treatedseparately. Thus, reference intervals for this and other species should always include information regardingthe method used for hemogram determination and the time elapsed since the blood collection. It is also suggested that the Drabkin's method may not be optimal for the evaluation of hemoglobin concentration in the mute swan blood stored for a longer time.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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