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Spleen mass as a measure of immune strength in mammals

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ABSTRACT

1. In studies of birds and their pathogens, spleen size has frequently been used to make inferences about immune system strength. However, the use of spleen size in mammals is more complicated because, in addition to having an immune function, the mammalian spleen is also a reservoir for red blood cells.

2. To assess the reliability of mammalian spleen mass as an indicator of immune activity, we quantified the white and red pulp mass by histology of spleens from shot red deer *Cervus elaphus*. We then analysed the relationships among spleen mass, the amounts of white and red pulp, and the deer's body condition relative to faecal counts of the nematode parasite *Elaphostrongylus cervi*.

3. White and red pulp mass were positively correlated so that an increase in spleen mass was a positive function of both components of the spleen. In male deer, which had significantly lower body condition and higher parasite loads than females, parasite counts were negatively correlated with spleen mass, white pulp mass, and red pulp mass.

4. Our findings suggest that (i) spleen mass in shot red deer is a reliable measure of white and red pulp content; and (ii) when looking at the red deer life history, which is greatly influenced by sex of the deer, splenic mass and white pulp mass could be used as reflections of immune system strength.

5. Future studies of mammalian spleens can contribute to the understanding of evolved strategies of immune response investment in mammals. However, determination of the white and red pulp spleen components using various sampling methods must be made prior to their application.

Keywords: body condition, histology, immune defence, red deer, sex

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INTRODUCTION

The ability of the immune system to respond to any challenge is vital for defence against pathogens. One of the most important components of the immune system in vertebrates is the spleen. The spleen is involved in maturation of splenic B-lymphocytes, in the production of immunoglobulins, and in lymphocyte (Type 2 T-help) mediated immune responses, in addition to being a site for identifying and filtering antigens (Kopp, 1990). In the last decade, interest in the size of immune defence organs as indicators of quality of the immune system has increased. In birds, splenic measurement studies have contributed to understanding evolved strategies of

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immune response investment. It is assumed that a larger spleen size implies a greater production of and storage of lymphocytes (e.g. Møller, Sorci & Erritzoe, 1998a; Smith & Hunt, 2004). Spleen size may reflect greater investment in immunity by healthier individuals and those in better condition, as the relative cost (resource mobilization relative to resource availability) for them is lower (Møller *et al*., 1998a). Alternatively, enlarged spleens in more heavily parasitized individuals could be a reaction to parasitism (increased production and storage of lymphocytes, pathological alterations) and this would be less dependent on the individual's nutritional condition (John, 1994). Much of the evolutionary context of spleen function and the relationship between spleen size or mass and immune function in mammals remain unclear due to the lack of evidence of functional relationships between spleen size and immune strength in birds (Brown & Brown, 2002; Smith & Hunt, 2004).

Some studies on rodents have found a positive relationship between parasite loads and/or disease and spleen size (Vincent & Ash, 1978; Garside, Behnke & Rose, 1989; Watkins *et al*., 1991). Spleen size in red deer *Cervus elaphus* may depend on body condition in addition to effects of variation between deer in resource allocation towards anti-parasite defence vs. growth (Vicente, Pérez-Rodríguez & Gortázar, 2007). These relationships with spleen size are sex and age-dependent. Sexual dimorphism in susceptibility to parasitic infections and disease is expected in species with polygynous mating systems (e.g. Moore & Wilson, 2002), due to the immunosuppressive effects of testosterone (Folstad & Karter, 1992), and/or differences in susceptibility to parasites caused by the different roles of sexes in activities related to sexual selection (Zuk, 1990).

The relative amount of red blood cells stored in the mammalian spleen is known to change depending on a number of variables and stimuli, such as stress levels or trauma induced haemorrhage. The red cell storage of the spleen is subject to short-term physiological variation arising from different causes: food ingestion, exercise, hypoxia, bleeding and decrease in blood pressure. For example, in humans up to 50% of spleen blood is actively expelled during exercise (Bakovic *et al*., 2005). This proportion is even higher in animals that depend on running to escape from predators. Fluctuating variability in the amount of red blood cells stored at any given moment may limit the use of splanchnometry (measurement of organ size and weight) to quantify or estimate the amount of immune defence tissue (Reilly, 1985). Variations in the red cell storage of the spleen can mask the true content of white cells when using whole spleen mass (SM) to estimate white cell amounts.

Briefly, on the basis of its gross appearance, the bulk of spleen parenchyma is referred to as red pulp (RP), with isolated areas of white pulp (WP) interspersed throughout. Microscopically, the RP appears red because it is formed from elements of the blood (mostly erythrocytes arranged into splenic cords and venous sinuses), which are not counted as immune defence tissue. The WP consists almost entirely of lymphocytes organized as periarteriolar lymphocyte sheath (PALS) where B-cells proliferate (Bacha, William & Wood, 1990). There is a connective tissue capsule around the spleen that is continuous with internal connective tissue septa that structures the spleen parenchyma. The connective tissue is interspersed with smooth muscle designed to, upon contraction, expel erythrocytes into circulation when needed, therefore making the proportion of red blood cells/RP in the spleen highly variable among individuals at any given time. To our knowledge, no attempts have been made to quantify the relative amount of WP mass in any mammal, or to assess the validity of SM as a measure of immune strength. The use of histological techniques would be very valuable to increase the understanding of the evolutionary role of defence against parasites in mammal hosts.

In this context, we hypothesized that WP mass may be an indicator of immune capacity, and, therefore, may correlate with parasite excretion levels in red deer faecal matter.

Investment in immune capacity may be different in males and females in polygynous species, like the red deer, because changes in survival, reproductive effort and reproductive expectancies differ between sexes and ages (Clutton-Brock, Guinness & Albon, 1982). Therefore, we accounted for sex variation in the spleen-body condition parasite relationships. We used histological techniques to quantify WP and RP mass in spleens from shot wild red deer. We then examined the relationships between spleen (SM, WP and RP) and body condition relative to faecal counts of a nematode, *Elaphostrongylus cervi*, known to commonly parasitize red deer.

MATERIALS AND METHODS

Deer were sampled across Ciudad Real province, South Central Spain (37°13′48″N–39°31′43″N; 06°34′06″W–2°25′54″W) after the rutting season from November to January (2000 to 2004) using 'monterías', large beats that involve several groups of dogs that drive the deer towards a line of hunters covering areas of over 400 ha. Post-mortem studies of 54 adult (>4 years; Klevezal & Kleinenberg, 1967), hunter-harvested deer were performed in the field. Total body length (to the nearest 0.1 cm) and sex were recorded ($n = 23$ females and $n = 31$ males). The spleens were collected and immediately transported to the laboratory, where the mass was measured (to the nearest 0.1 g). Body condition was estimated using the kidney fat index (KFI) (Finger, Brisbin & Smith, 1981), which is defined as weight of the kidney fat in relation to kidney weight (%).

The nematode *E. cervi* is widespread in Spanish populations of red deer (Vicente, Fernandez de Mera & Gortazar, 2006). Adult *E. cervi* are found in the fascia and connective tissue around skeletal muscles. Heavily infected animals may develop respiratory distress and, rarely, may develop neurological signs (Watson, 1984). After travelling up the bronchial tree, first-stage *E. cervi* larvae (L1) are coughed up and swallowed, allowing for dispersal into the host's gastrointestinal tract and, eventually into the host faeces. Fresh faecal samples were collected from the rectum of the shot deer; L1 were extracted, identified and expressed as number of larvae per gram of faeces (Forrester & Lankester, 1997). The sampling period was limited to winter in order to avoid seasonal variations in parasite excretion (Vicente, Fierro & Gortazar, 2005).

Spleen tissue samples were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax and sectioned at 4 μm then were stained with haematoxylin-eosin and examined by light microscope (Bacha, William & Wood, 1990). WP was considered equivalent to the lymphocyte population of the spleen (PALS), whereas RP was considered to be everything else (the splenic cords and the sinuses). Quantitative measures of the presence of RP and WP were estimated by separately examining four different fields of the stained slides at 40× magnification. Each of the four fields was individually divided into 100 different sampling points by a 1 cm^2 eye piece graticule divided into 10×10 squares. These divisions represented a sufficient and adequate number of measurements to obtain reproducible results (Varas, Torroba & Zapata, 1992). The presence of WP and RP tissues in each of the 100 squares was recorded. The amount of red and WP were then expressed them as a proportion of the spleen, which was used as a correction factor to estimate WP and RP (given a known SM).

We used general linear models to examine (i) sex differences in KFI, parasite counts, SM, RP and WP while controlling for body size; (ii) sex differences in the proportion of RP and WP; and (iii) the bivariate interactions between SM, RP and WP while controlling for body size, respectively, and separately for each sex. To assess the use of SM as a measure of immune strength, we used path analysis or causal modelling (Mitchell, 1992). This statistical method allowed for an understanding of comparative strengths of direct relationships and

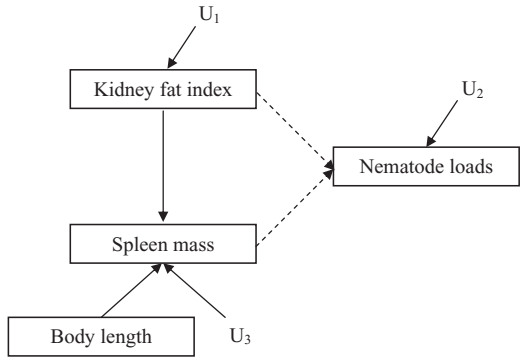

Fig. 1. Hypothesized path diagram. Lines denote positive effects, dashed lines denote negative effects. U represents latent variables (unexplained variance of the variable receiving the arrow).

mediated pathways among the set of variables. The proposed structured relationships (pathways) of spleen size and KFI relative to counts of *E. cervi* L1 are displayed in Fig. 1. In the path diagram, pathways represent the proposed hypotheses, with the goal being to build a path diagram that fits the data. Path analysis can test complex relationships between variables in one step (variables can have a causal effect on a set of variables and at the same time experience the effects of others), quantifying how well the data fit a set of hypothesized structured relationships between variables. Path diagrams consist of variables connected by arrows, representing directed relationships. These variables can be manifest (quantified and included explicitly in the model), or latent (proposed to exist and not quantified). We separately assessed the path diagrams for SM, WP and RP. Goodness of fit of our models to the data was assessed by the χ^2 and Bentler-Bonett tests. *E. cervi* L1 counts + 1, KFI, SM, RP and WP were $\log_{10}$ transformed prior to analysis. Values for phenotypic traits are expressed as mean ± S.E. (95% CI).

RESULTS

Mean abundance of *E. cervi* L1 counts, mean SM and mean KFI across sex classes are shown in Fig. 2. Male red deer presented statistically higher parasite counts ($F_{1,52} = 6.1$, $P = 0.02$) and lower body condition scores than females ($F_{1,52} = 29.3$, $P < 0.001$). SM ($F_{1,51} = 0.89$, $P = 0.34$, Fig. 2), RP ($F_{1,51} = 1.1$, $P = 0.29$) and WP ($F_{1,51} = 0.0$, $P = 0.98$) did not differ between sexes after controlling for the effect of body size ($P > 0.05$ always). Males and females had a similar proportion of WP ($F_{1,51} = 0.51$ $P = 0.48$; males 12.9 ± 0.9%, females 12.7 ± 0.9%); the WP proportion was not affected by the SM ($F_{1,50} = 1.68$, $P = 0.20$) after controlling for body size ($F_{1,50} = 2.01$, $P = 0.16$). Therefore, as would be expected, males and females presented similar proportions of RP ($F_{1,51} = 0.51$, $P = 0.48$; males 87.1 ± 0.9%, females 87.3 ± 0.9%).

White pulp and RP were positively correlated with each other ($F_{1,28} = 5.1$, $P = 0.03$ and $F_{1,20} = 4.7$, $P = 0.04$, for males and females, respectively) after the effects of body mass were taken into account (for the effect of body mass on WP and RP, P was always >0.05). Therefore, an increase in SM was a positive function of both WP ($F_{1,27} = 26.7$, $P < 0.001$, SM = 0.39WP + 1.89, $R^2 = 0.51$; and $F_{1,19} = 6.5$, $P = 0.02$, SM = 0.44WP + 1.69, $R^2 = 0.52$, for males and females, respectively) and RP ($F_{1,27} = 556.7$, $P < 0.001$ and $F_{1,19} = 1103.5$, $P < 0.001$, for males and females, respectively) after controlling for the effect of body size (for the effect of body mass on SM, P was always >0.05).

The path analyses, the probability values, the significant value for the directed relationships and the goodness of fit of the solved path diagrams across sexes are shown in Table 1. Our proposed models do not show a significant lack of fit. No statistically significant relationships

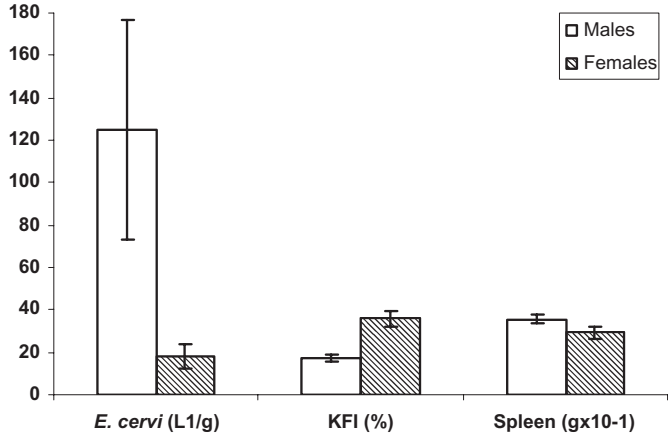

Fig. 2. Mean *E. cervi* abundance [$\log_{10}$ (*E. cervi* L1 count + 1)], body condition (KFI, %) and spleen mass across sexes ($n = 31$ males and 23 females). KFI, kidney fat index.

Table 1. Path diagram statistics

Directed relationships	Overall spleen mass		Red pulp mass		White pulp mass	
Fit statistics	M	F	M	F	M	F
KFI → spleen	0.032	–0.014	0.003	–0.021	0.218	0.054
KFI → *E. cervi*	–0.307	0.819	–0.415	0.823	–0.105	0.77
Spleen → *E. cervi*	–4.278*	0.589	–4.001*	0.510	–1.630*	0.637
Body length → spleen	0.001	0.004	0.001	0.003	0.003	0.009
Chi^2	0.20	0.98	0.25	0.97	0.22	0.95
Bentler-Bonet	0.81	0.98	0.92	0.98	0.72	0.98

KFI, kidney fat index.
Probability values and significant value for the directed relationships included in the path analyses (*$P < 0.001$). Goodness of fit of the models were tested by (i) The chi-squared test; and (ii) the Bentler-Bonett normed-fit index. *P*-values for chi-squared test was ≥0.05; thus there are no significant differences between the real data and the models proposed. Bentler-Bonett normed-fit index approaches 1 in value as fit becomes perfect.

between SM and KFI, *E. cervi* and KFI, or body length and SM were detected. Parasite counts were negatively related to SM, WP and RP in males. However, no significant relationships were found for females.

DISCUSSION

To date, the assumption that a larger spleen implies a larger production or storage of lymphocytes in mammals (Møller *et al.*, 1998b; Møller, Sorci & Erritzoe, 1998a) has not been established. This may partly be because the red blood cell content, which may be subject to short-term variations in volume, is an important task of the mammalian spleen, and, as a result, a major driver of SM variations. This is the first time a study has been performed to examine the possibility of using SM as a reliable estimate of the amount of lymphoid tissue in the spleen, and, to examine whether or not changes in the whole SM reflect proportional changes in both white and RP. Our findings suggest that splanchnometry of the spleen assessed by histology can contribute to the understanding of immune response strategies in mammals.

We found a relatively constant proportion of WP compared to the whole spleen and an increase in SM due to both WP and RP increase. We demonstrated that in red deer under our sampling conditions (strenuous exercise and shot bounded), SM is a good indicator of WP. The sampling conditions in our study animals, shot during an intense exercise, and probably experiencing a high red blood cell ejection from the spleen; could compare to field samplings of other mammal species occurring after physical restraint, intense exercise and after stressful situations, such as hunting. In particular, the admixture of muscle in the spleen capsule is very pronounced in ruminants and horses because they depend, primarily, on running for their survival, meaning that they can store erythrocytes in the spleen and release them into the general circulation when needed for extra oxygen carrying capacity. Therefore, measuring SM in deer may be especially reliable if they are exercised first so that blood is ejected from the spleen. In either case, we strongly recommend SM and its components to be individually assessed by using histology, or other reliable technique.

The analyses of structured relationships of SM and KFI relative to counts of *E. cervi* yielded that in males, SM, WP and RP were negatively correlated with the parasite load. Females presented much lower *E. cervi* L1 counts than males. Sexual dimorphism in susceptibility to parasitic infections and disease is expected in species with a polygynous mating system (e.g. Moore & Wilson, 2002). This is possibly due to the immunosuppressive effects of testosterone (Folstad & Karter, 1992), and/or differences in the susceptibility to parasites caused by the different roles of sexes in activities related to sexual selection (Zuk, 1990). Our findings are compatible with red deer life-history, which is greatly influenced by sex. Although the RP, the WP and the proportion of WP did not differ between sexes, there were negative correlations between SM, WP and RP with the parasite loads in males, but not in females. This may be because SM may indicate immune investment in males, the more stressed sex during the sampling season, just after rutting. Adult males are involved in fighting and holding harems of hinds during mating season (Clutton-Brock *et al.*, 1982). This increased activity level during mating season and after rutting can cause individuals experience high level of testosterone and may even deplete body reserves to levels of starvation. Therefore, at the season of the year when samples were collected, males were more likely to experience a trade-off between investing resources in immune response or to reproductive activities. Our results suggest that males in better condition (higher KFI) may have been able to develop and/or maintain heavier spleens (with larger total amount of WP) and therefore may have been able to maintain lower levels of infection by *E. cervi* (Vicente *et al*., 2007).

Parasites may cause an increase in spleen size in infected individuals because of an increased immune response expected in parasitized deer. Studies in mammals, mostly in rodents, have found a positive relationship between parasite loads or disease and spleen size (Vincent & Ash, 1978; Garside *et al.*, 1989; Watkins *et al*., 1991). Nevertheless, our negative relationship between spleen size and *E. cervi* counts may suggest that if any causal relationship exists, *E. cervi* larvae excretion would be determined (at least partially) by SM and not vice versa (as may have been inferred if we had found a positive relationship) as a result of greater investment on immunity by healthier and/or higher quality individuals. In this sense, Vicente *et al*. (2007) found a positive relationship between KFI and SM in red deer from our study area using a bigger sample size. Therefore, SM could reflect immune capacity in red deer rather than immune system reactivation, and individuals with larger spleens are apparently more capable of maintaining lower parasite levels. We note that we present a correlational study, and the question of whether spleen size is a cause or a consequence or both, as there may be a feedback process, of parasite loads, could vary according to the nature of the host-parasite system, and is an interesting subject that needs to be explored.

In conclusion, by using histological techniques, we have shown that mammal spleen size, besides being influenced by its role as a reservoir of red blood cells, may be a good estimate of the amount of lymphoid tissue in the organ. Furthermore, by exploring the relationships with sex, body condition and the levels of infection of the nematode *E. cervi*, we have demonstrated that SM and its content in lymphoid tissue may be useful indices of immune function in wild mammals.

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REFERENCES

Bacha, W.J. Jr, William, J. & Wood, L.M. (1990) *Color Atlas of Veterinary Histology*. Lea & Febiger, Philadelphia, PA, USA.

Bakovic, D., Eterovic, D., Saratlija-Novakovic, Z., Palada, I., Valic, Z., Bilopavlovic, N. & Dujic, Z. (2005) Effect of human splenic contraction on variation in circulating blood cell counts. *Clinical and Experimental Pharmacology and Physiology*, **32**, 944–951.

Brown, C.R. & Brown, M.B. (2002) Spleen Volume varies with colony size and parasite load in a colonial bird. *Proceedings of the Royal Society B*, **269**, 1367–1373.

Clutton-Brock, T.H., Guinness, F.E. & Albon, S.P. (1982) *Reed Deer: Behaviour and Ecology of Two Sexes*. University of Chicago Press, Chicago, USA.

Finger, S.E., Brisbin, I.L. Jr & Smith, M.H. (1981) Kidney fat as a predictor of body condition in white tailed deer. *Journal of Wildlife Management*, **45**, 964–968.

Folstad, I. & Karter, A.J. (1992) Parasites, bright males, and the immunocompetence handicap. *American Naturalist*, **139**, 603–622.

Forrester, S.G. & Lankester, M.W. (1997) Extracting Protostrongylid nematode from ungulate faeces. *Journal of Wildlife Diseases*, **33**, 511–516.

Garside, P., Behnke, J.M. & Rose, R.A. (1989) The immune response of male DSN hamsters to a primary infection with *Ancylostoma ceylanicum*. *Journal of Helminthology*, **63**, 251–260.

John, J.L. (1994) The avian spleen: a neglected organ. *Quarterly Review of Biology*, **69**, 327–351.

Klevezal, G.A. & Kleinenberg, S.E. (1967) *Age Determination of Mammals from Annual Layers in Teeth and Bones*. Institute for Applied Technology, Springfield, USA.

Kopp, W.C. (1990) The immune functions of the spleen. In: *The Spleen: Structure, Function and Clinical Significance* (Ed. by A. J. Bowdler), pp. 103–126. Chapman & Hall Medical, London, UK.

Mitchell, R.J. (1992) Testing evolutionary and ecological hypothesis path analysis and structural equation modelling. *Functional Ecology*, **6**, 123–129.

Møller, A.P., Sorci, G. & Erritzoe, J. (1998a) Sexual dimorphism in immune defence. *American Naturalist*, **152**, 605–619.

Møller, A.P., Christe, P.H., Erritzoe, J. & Mavarez, A. (1998b) Condition, disease and immune defence. *Oikos*, **83**, 301–306.

Moore, S.L. & Wilson, K. (2002) Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, **297**, 2015–2017.

Reilly, F.D. (1985) Innervation and vascular pharmacodynamics of the mammalian spleen. *Cellular and Molecular Life Sciences*, **41**, 187–192.

Smith, K.G. & Hunt, J.L. (2004) On the use of spleen mass as a measure of avian immune system strength. *Oecologia*, **138**, 28–31.

Varas, A., Torroba, M. & Zapata, A.G. (1992) Changes in the thymus and spleen of the turtle Mauremys caspica after testosterone injection: a morphometric study. *Developmental and Comparative Immunology*, **16**, 165–174.

Vicente, J., Fierro, Y. & Gortazar, C. (2005) Seasonal dynamics of the fecal excretion of *Elaphostrongylus cervi* (Nematoda, Metastrongyloidea) first stage larvae in Iberian red deer (*Cervus elaphus hispanicus*) from South Spain. *Parasitology Research*, **95**, 60–64.

Vicente, J., Fernandez de Mera, I.G. & Gortazar, C. (2006) Epidemiology and risk factors analysis of elaphostrongylosis in red deer (*Cervus elaphus*) from Spain. *Parasitology Research*, **98**, 77–85.

Vicente, J., Pérez-Rodríguez, L. & Gortázar, C. (2007) Sex, age, spleen size, and kidney fat of red deer relative to infection intensities of the lungworm *Elaphostrongylus cervi*. Naturwissenschaften, **94**, 581–587.
Vincent, A.L. & Ash, L.R. (1978) Splenomegaly in jirds (*Meriones unguiculatus*) infected with *Brugia malayi* (Nematoda: Filaroidea) and related species. *American Journal of Tropical Medicine Hygiene*, **27**, 514–520.
Watkins, R.A., Moshier, S.E., Odell, W.D. & Pinter, A.J. (1991) Splenomegaly and reticulocytosis caused by *Babesia microti* infections in natural-populations of the montane vole, *Microtus montanus*. *Journal of Protozoology*, **38**, 573–576.
Watson, T.G. (1984) Tissue worm in red deer. Symptoms and control. In: *Aglink Farm Production and Practice 249*, pp. 1–2. Ministry of Agriculture and Food New Zealand, Wellington, New Zealand.
Zuk, M. (1990) Reproductive strategies and sex differences in disease susceptibility: an evolutionary viewpoint. *Parasitology Today*, **6**, 231–233.

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