**Dissolved oxygen regimen (PO₂) may affect osmo-respiratory compromise in European sea bass (Dicentrarchus labrax, L.)**

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**Abstract**

Fundamentally, in land based Mediterranean aquaculture, two techniques are applied to supply water with oxygen: puddling water aeration and application of pure oxygen. The two oxygenation techniques result in quite different PO₂ regimens and, consequently, different fish growth performance and gill morphology.

Data exist showing a reduction in total respiratory surface (RSA) and increasing gas diffusion distance (GDD) in gills of sea bass (Dicentrarchus labrax, L.) farmed under elevated PO₂ regimens. That such a modification might have an effect on the ion regulation has been defined elsewhere as osmoregulatory compromise.

In this study, European sea bass previously acclimatized to two PO₂ regimens, mild hypoxia and mild hyperoxia (70-80% and 130-140% of the saturation value, respectively), were challenged for 1 hour with hypo-osmotic plus Na+ loss was; respectively. For our data are compatible with the hypothesis of an osmotic advantage of sea bass exposed to an elevated PO₂ regimen, achievable with application of pure oxygen, instead of simple water aeration.

**Introduction**

European sea bass (Dicentrarchus labrax, L.) is a largely euryhaline species that can be found in truly fresh water, brackish water, normal sea water, or very highly saline water (up to 90 ppt) and it is one of the most important species for Mediterranean aquaculture. Several different technologies are used to farm this species and include extensive farming in large brackish water ponds (Valliculture) and coastal lagoons and intensive farming in land-based raceways and coastal pen cages (Barnabé, 1990; Cataudella and Bronzi, 2001). Water-recirculation technology was developed to farm sea bass in inland areas with artificial salted water within the range of 8 to 20 ppt.

The oxygenation techniques applied in land-based intensive farming of this species include either puddling water aerators or dissolving pure oxygen in water with on-purpose-made machines (Saroglia, 2001). It is very well known by stakeholders that with puddling water aerators, the PO₂ regimens range among values corresponding to 30-85% of the saturation values, whereas with the application of pure oxygen the regulation systems often ensure values ranging from 110 to 140% of the saturation values (Barnabé, 1990). Important differences in production quality have been reported as a result of the water oxygenation technology applied (Landoli et al., 1995) and significant differences in sea bass gill morphology have been described as a result of the water PO₂ regimens (Saroglia et al., 2000, 2002).

Fish gills are responsible for several physiological functions that require anatomical and physiological compromises (Randall and Daxboeck, 1984; Olson, 1991; Perry and McDonald, 1993). The rate of gas transfer through fish gills is regulated by Fick’s diffusion equation, as reported by Jobling (1994), and the basic conflict between gas exchange and ion regulation in the gills of freshwater fishes has been described by Randall et al. (1972). The latter authors indicated that a large, permeable gill membrane is required for efficient gas transfer but that a small, impervious epithelium is needed to minimize diffusive ion losses. These authors demonstrated that rainbow trout accelerate Na⁺ loss with an increase in oxygen consumption during exercise, which they attributed mainly to an increased functional surface area (FSA) of the gills during activity.

As suggested by Nilsson (1986), the balance between ‘need of oxygen’ and ‘need of osmotic regulation’ has been defined as osmoregulatory compromise. There are two major scenarios in which the osmoregulatory compromise may interfere with osmotic homeostasis: when oxygen demand is high, and gill perfusion must be increased to favour gas exchange at the expense of ion regulation; and when the epithelium must be thickened to defend ion balance at the expense of gas exchange. Within a species, changes in environmental conditions, such as elevated temperature or salinity, and exercise, serve to unmask the nature and degree of the osmoregulatory compromise (Sardella and Brauner, 2007). In general, elevated metabolism associated with high temperature, result in a partial loss of osmoregulatory control and in other cases, exposure to condition requiring an upregulation of osmoregulatory characteristic of the gills (e.g., exposure to dilute water) can result in morphological changes in the gill that impair gas exchange (Henriksson et al., 2008).

The oxygen demand and PO₂ fluctuation could be compensated by adapting the total
respiratory surface (RSA), as well as Functional Respiratory surface (FSA), and gas diffusion distance (GDD) in water. In fact, a positive correlation was found in sea bass (*D. labrax*, L.) between environmental oxygen and both GDD and RSA (Saroglia et al., 2000, 2002, 2007). Sea bass reared under hyperoxia exhibited significantly higher GDD than fish reared under normal oxygen concentrations. This also occurred when improvement in O2 solubility was due to a seasonal water temperature decrease. In their conclusions, Saroglia et al. (2002), suggested that the application of pure oxygen in intensive aquaculture represents an energy advantage for osmoregulatory mechanisms.

However, such sophisticated compensatory mechanisms likely require energy, too. The increase in FSA causes a rise in water loss and ionic gain through the gills in fishes in a hypertonic environment such as salt water (Evans, 1993). Fishes respond to this by increasing their water intake, which requires an increase in energy in order to excrete the excess ions through the gills. In contrast, a higher ion loss occurs when fish are acclimated to a hypotonic environment such as freshwater and respond with adequate mechanisms consisting in an enlarged water uptake and elimination of excess water and ions via the kidney. So far, a demonstration of the effects that the PO2 regimen may have on fish osmotic control is lacking. The aim of this study was to verify the hypothesis that an increase in PO2 actually provides an advantage for ion regulation in fish.

**Materials and methods**

**First acclimatization facilities**

All the trials were conducted on European sea bass (*Dicentrarchus labrax*, L.) obtained from the commercial hatchery Nuova Azzurro in Civitavecchia (Rome) and farmed for an acclimatization period (3 to 6 months) at low temperatures in Civitavecchia (Rome) and farmed for an acclimatization period (3 to 6 months) at low temperatures. Fishes were kept in 4 m^3^ fiberglass tanks connected to a water recirculation system providing about 24 water refilings per day, at a water temperature of 20±1°C, salinity 10 g/L, dissolved oxygen (PO2) 95-105% of the saturation value, total gas pressure ±10%, free CO2<15 mg/L, NH4<0.03 mg/L, NO3<20 mg/L, pH 7.2, and alkalinity 140-200 mg/L CaCO3. The photoperiod was constant at 10L:14D and the fish were fed twice a day with extruded commercial marine fish food (Hendrix s.p.a., Nuetroc group, Verona, Italy). When needed, fish were randomly sampled and transferred to adequate tanks, where experimental trials were conducted.

**First trial**

The first trial consisted in measuring the rate of the Na+ loss at different times, when sea bass acclimatized to different PO2 levels were exposed to a hypo-osmotic challenge. For this purpose, 40 fish ranging from 300 to 400g individual weight, divided in two groups of 20 each and acclimatized as above, were transferred to two 250-L fiberglass tanks with an initial density of <30 kg/m^3^. The environmental conditions were the same as previously described, with the exception of PO2, which was either 70-80% (hypoxia treatment) or 130-140% (hyperoxia treatment). In the latter case, total gas pressure ranged from 98 to 102% of the saturation (Novatec® 3000 ten- simeter). The desired PO2 conditions were ensured by blowing pure oxygen in the system, at the pipe entering the pump. Feeding regime consisted of Nutreco Perla® extruded pellets, delivered with an automatic tape-feeder at a maintenance ratio. After 60 days, during which no fish died, nine fish from each of the two PO2 conditions were randomly caught and submitted, at the same temperature, to hypo-osmotic stress. For the challenge, fish skin was previously rinsed with de-ionized water made by adding CaCl2 to de-ionized water with the pH buffered to 7.2 (±0.05 mmol L^-1^ Na^+^ and Cl^−^, 0.25 mmol L^-1^ Ca^2+^, pH 7.5, hardness 10 mg/L as CaCO3) and normoxic PO2, basically according to the protocol of Gonzalez and McDonald (1994). For the challenge, fish skin was previously rinsed with de-ionized water and thereafter 9 fish were randomly put into 3 jars sizing 20 L volume, 3 fishes per jar. The jars were filled with exactly 5 L of water. A 100-mL water sample was collected from each jar immediately after introducing the fish in order to determine the Na+ molarity in the water at time t0. During the osmotic challenge, fishes were exposed to a manipulation treatment as well, by manually stirring the jars, in order to magnify the ion losses by increasing blood cateholamines and metabolism. After 5, 10, 30, and 60 min, the same volume of water was sampled to determine Na+ molarity. In computing the results of the analyses, the residual water volume was corrected for the sampled amount. At the end of the trial each group of three fishes was weighed individually to calculate Na+ efflux per minute and per gram of fish. The average weight of fishes was 393.33±5 and 415.67±7.1 g among the hyperoxia and hypoxia populations, respectively (differences at P=0.33 were not significant).

**Second trial**

The second trial consisted in the assessment of Na+ and Cl− efflux in sea bass acclimatized under two PO2 regimes as in the first trial when exposed to a hypo-osmotic challenge for 60 min. For this purpose, two groups of 40 fishes each acclimatized as above, with an initial body mass ranging from 45 to 55 g, were randomly selected to be transferred to two 250-L fiberglass tanks where environmental conditions were the same as in the first trial, including the PO2 regimes. After 60 days, during which no fish died, 10 fishes from each of the two PO2 conditions were randomly caught and submitted, at the same temperature, to hypo-osmotic stress. Freshwater was prepared as in the first trial; however, after washing and drying, each fish was separately introduced into a jar filled with exactly 2 L water for the challenge. A manipulation treatment as well was performed, by manually stirring the jars, in order to magnify the ion loss by increasing blood cateholamines and metabolism. After 60 min of treatment, the fishes were sampled and weighed individually; the average weight was 64.52±13 and 62.48±12 g among the hyperoxia and hypoxia populations, respectively (differences at P=0.78 were not significant). In three fishes per PO2 population, blood samples (about 150 μL) were promptly collected from the caudal vein with a heparinized needle and glass microtube (microsampler AVL®, Roswell, GA, USA) and immediately analyzed with a blood gas analyser (AVL®, mod. OPTI 2) to determine Na+, K+, pH, PO2, PCO2, TCO2, HCO3, Hb, and % hematocrit. From the 1.9 L of residual water in each jar, a 100-mL sample was collected for ion analyses.

**Water analyses**

All water samples were immediately stored at -20°C until the analyses. Ionic molar concentrations were determined by ionic chromatography with DIONEX DX500-DX600 and the total amount of Na+ and Cl− in the water was measured with CHROMELON BIONEX 1999 software, version 6.11, build 490. The ion loss from the fishes in 60 min was calculated for each jar as the difference between the water ion content at the end and at the beginning of the test and dividing the obtained value by the body mass. The average ion loss, expressed as mmol g^-1^ min^-1^, was calculated for each fish population.

**Statistical evaluations**

Results were statistically evaluated, with the SigmaStat® programme, by one-way analysis of
Results and discussion

The Na⁺ loss at different time intervals during a 60-min hypo-osmotic challenge is reported in Figures 1 and 2. The ion efflux during the first trial was shown to be, in a 60 min, 3.74 μmol g⁻¹ h⁻¹ and 2.06 μmol g⁻¹ h⁻¹ for fish previously acclimatized under conditions of hypoxia and hyperoxia, respectively (Figure 1). Therefore, fishes acclimatized at low PO₂ lost Na⁺ 1.8 times faster than fishes acclimatized under hyperoxia. Gonzalez and McDonald (1992) reported for rainbow trout, immediately after epinephrine infusion, a Na⁺ loss that, if sustained, would approach 21% of total body Na⁺ per hour. In our study, Na⁺ efflux resulted in a rate of 163.72 and 112.23 nmol g⁻¹ min⁻¹, from the hypoxia and hyperoxia groups, respectively, during the first 5 min of the trial (Figure 2); if sustained, this would approach 15.3 and 11.2% of total body Na⁺, in the same order of magnitude (assuming a total body Na⁺ as 0.06 nmol g⁻¹, unpublished data). Actually, in 1 hour, the total loss approached 6.2% and 3.4% of total body Na⁺. Again, this is in the order of magnitude of the 3% of the total body Na⁺ per hour reported by Gonzalez and McDonald (1992) for rainbow trout during exercise.

As reported in Table 1, a 60-min ion loss from the second trial resulted in a rate of 76.86±12 and 179.28±32 nmol g⁻¹ min⁻¹ for Na⁺ in the fish populations previously acclimatized to the hyperoxia and hypoxia regimens, respectively, approaching in 1 hour 9.6% and 18.4% of total body Na⁺. For Cl⁻ this loss was 62.02±11 and 157.28±28 nmol g⁻¹ min⁻¹. According to statistical analyses, the rate differences among populations are highly significant for both ions (P<0.001). The fact that the loss rates from the two trials do not correspond exactly can be mainly explained by the fact that different sizes may result in a different ion loosing (inversely proportional with sizes as it is resting metabolism) and in the second trial sizes were smaller than in the first one. Also, we could assume that the associated manipulation stress could not be perfectly repeated in the two trials.

The focus of the present study being on the relationship between the previous PO₂ acclimatization regimen and ionic loss at the gills, we have actually measured flux across the whole animal. Even though the ionic loss through the skin may be considered negligible, the kidneys contribute to ionic loss (McDonald and Wood, 1981). However, it has been estimated in rainbow trout during exercise that an hourly basis urinary Na⁺ excretion is unlikely to exceed 1.0 nmol g⁻¹ min⁻¹ (Gonzalez and McDonald, 1992), which is more than 100 times less than the Na⁺ efflux found in this experiment. This confirms that the ionic loss was occurring predominantly across the gills. We also measured increased concentrations of Na⁺ and Cl⁻ in the jars, instead of the real ionic flow; therefore, the results should be interpreted as a balance between the influx and the efflux from fish to water. Nevertheless, the water utilized for the challenge was almost Na⁺ and Cl⁻ free, so we may reasonably assume the Na⁺ and Cl⁻ influx to be negligible. Gonzalez and McDonald (1992) previously described the same experimental procedure; however, they used freshwater fishes to avoid the problem of salinity transfer. In our case, sea bass that were previously acclimatized to 10 g/L salinity were transferred to freshwater and that may have resulted in an even more severe stress; nevertheless, the range of ionic loss recorded in sea bass showed the same order of magnitude of the findings by previous authors.

Evidence of a PO₂-controlled osmo-regulatory advantage

This study shows an important relationship between PO₂ and the efflux of Na⁺ and Cl⁻ in fishes exposed to a hypo-osmotic challenge together with manipulation stress; the Na⁺ and Cl⁻ efflux was elevated 1.9- and 2.5-fold, respectively, in fishes previously acclimatized at the low PO₂ conditions. This can be explained by the studies of Saroglia et al. (2000) and Saroglia et al. (2002) in which the experimental conditions, including the level of oxygen and exposure duration, were comparable to ours. They reported that hyperoxia is associated with discrete changes in morphology of the gill, including reduced RSA and increased GDD in the gills of sea bass; the reduced attitude to exchange gases with water (O₂ and CO₂) also caused a reduction in the ion efflux rate. The manipulation stress increased the catecholamine levels, as this condition probably magnifies the ion efflux by increasing intralamellar pressure; as also interpreted by Gonzalez and McDonald (1992), injecting epinephrine in rainbow trout achieved the same effect.

Exposure to hyperoxia, as well as hypercapnia, is reported to elevate the partial pressure of CO₂ in the blood (PCO₂), resulting in a respiratory acidosis (Höbe et al., 1984, cit. in Brauner et al., 2000). The respiratory acidosis is compensated within 24-96 h, predominantly through an increase in plasma HCO₃⁻ and an associated reduction in plasma Cl⁻ (Wood and Jackson, 1980; Wheatly et al., 1984). Brauner (1999), as well as Brauner et al. (2000), reported on a dramatic change in acid-base equivalent exchanges across the gills during hyperoxia exposure in freshwater fishes and also found that hyperoxia impaired hypo-osmoregulation following seawater transfer of Atlantic salmon (Salmo salar) smolts. Actually, the latter authors exposed fishes to elevated hyperoxia for a short period, during the transport only, whereas in our study the long exposure of sea bass to mild hyperoxic conditions in brackish water could be expected to result in a physiological equilibrium. Nevertheless, our blood
analyses were performed after only 1 hour of the successive hypo-osmotic plus manipulation stress; thus, new compensation dynamics might have started. No significant differences were recorded in the blood Na⁺ and Cl⁻ among sea bass from the two experimental groups; nevertheless, a tentative trend was observed for reduced Cl⁻ in the fish exposed to hyperoxia, associated with a relatively significant hypercapnia, higher oxygen concentration, and lower pH, whereas no changes were seen in HCO₃⁻ and total CO₂. Again, in spite no significant differences were reported, a tentative trend for lower tot. Hb and Htc% values in the experimental group maintained under hypercapnia, higher oxygen concentration, whereas the blood pH was only significantly reduced by the hypoxia condition and not in the range from “normoxia” to the highest hyperoxia experienced. In contrast, plasma bicarbonate and P⁰₂ increased with increasing salinity on physiology and behavior also have to be taken into account including, among others, tissue permeability to water and ions, gill ventilation, perfusion, and functional surface area (Swanson, 1998).

It can be also argued that the different oxygen concentrations may cause an acid-base disturbance, affecting blood pH and gill morphology. In this regard, Cecchini and Caputo (2003) published a study on the acid-base balance in sea bass in relation to water oxygen concentration. The study was carried out for 5 weeks under controlled conditions, the P⁰₂ conditions being assayed at 64, 97, 150, and 250% of the saturation values and at a salinity and temperature similar to those in our experiments. In spite the authors included a quantitatively minor error in calculating the average values of the pH, it is evident from their data that no significant differences in sodium concentrations and blood P⁰₂ were existing, whereas the blood pH was only significantly reduced by the hypoxia condition and not in the range from “normoxia” to the highest hyperoxia experienced. In contrast, plasma bicarbonate and P⁰₂ increased with increasing environmental P⁰₂. Nevertheless, the differences in the reported acid-base values only affected the P⁰₂ range to a low degree, corresponding to our environmental conditions. Although we cannot completely exclude a limited overlapping of the acid-base conditions on the direct effect of P⁰₂ from our data, the differences in the ion regulation are a consequence of the water oxygenation technology applied. **Energy aspects**

The overall picture of osmotic regulation for both freshwater and saltwater fish species is reasonably clear since the osmoregulatory organs and tissues have been identified: digestive tract, gill, kidney, urinary bladder, and liver as ureotelic regulators. Both passive and active flux of solutes, mainly NaCl plus urea, in ureotelic tissues have been assessed in many species. The chloride cell, or mitochondria-rich cell, in the teleost gill has been shown to play an important role in ionic homeostasis by compensating passive epithelial ion flux through appropriate active ion uptake and excretion (Evans, 1993). Interactive effects of salinity on physiology and behavior also have to be taken into account including, among others, tissue permeability to water and ions, gill ventilation, perfusion, and functional surface area (Swanson, 1998).

The question is: how much of the metabolic oxygen (MO₂) is specifically dedicated to regulating the balance between minerals and water?

Boeuf and Pajan (2001) report conflicting information from the literature regarding the metabolic cost of exposure to different salinities. On one hand, a drastic reduction in the metabolic rate of 20-28% at an isotonic salinity relative to that in freshwater and/or saltwater was reported (Rao, 1968). Such studies support the hypothesis that the energy cost of osmoregulation is lower in an isosmotic medium, where the gradients between blood and water are minimal, and that these energy savings are substantial enough to increase

### Table 1. TRIAL 2: Na⁺ and Cl⁻ release in water by sea bass acclimatized at two different P⁰₂ conditions, during a 60-min osmotic challenge. Ion concentrations in water are reported at the beginning of the test and after 60 min; water jar volumes were initially 2 L.

| Ion concentration in water at “t0”, mg.L⁻¹ | Hyperoxia | Hypoxia |
|------------------------------------------|-----------|---------|
| Na⁺                                      | 0.16±0.04 | 0.22±0.13¹ |
| Cl⁻                                      | 7.00±1.12 | 8.74±1.47¹ |
| Fish weight (n=10), g                     | 64.5±13.8 | 62.4±12.4 |
| Ion release rate, nmol.g⁻¹.min⁻¹           | 76.86±12.79 | 62.02±11.16¹ |

All the data represent the average of 10 replicates. Comparing boxes reporting for the same ion in water, as well as for ions release, different letters indicate significant differences (P<0.001).

### Table 2. TRIAL 2: Trend on concerning blood parameters in D. labrax, previously acclimatized at two different P⁰₂ conditions, after a 60-min osmotic challenge.

| Fish | 1 | 2 | 3 | mean±SD | 11 | 22 | 33 | mean±SD |
|------|---|---|---|---------|----|----|----|---------|
| Na⁺  | 128.8 | 116.5 | 124.3 | 123.2±1.9 | 113.5 | 121.3 | 112.5 | 115.7±4.8 |
| K⁺   | 1.59 | 2.58 | 1.6 | 1.92±0.6 | 2.07 | 2.39 | 3.13 | 2.33±0.5 |
| Cl⁻  | 104.5 | 99.1 | 100.5 | 101.36±2.8 | 92.3 | 100.9 | 78.6 | 90.6±11.2 |
| pH   | 7.65 | 7.46 | 7.71 | 7.62±0.14 | 7.52 | 7.40 | 7.42 | 7.45±0.07 |
| P⁰₂  | 15.6 | 11.4 | 13.6 | 13.60±2.2 | 13.6 | 23.1 | 27.7 | 21.4±7.2 |
| P⁰₂CO₂ | 10.4 | 11.8 | 10.4 | 10.86±0.8 | 12.4 | 19.0 | 14.7 | 15.3±3.3 |
| T⁰₂CO₂ | 13.9 | 10.9 | 15.9 | 13.56±2.5 | 12.6 | 15.3 | 12.3 | 13.6±1.6 |
| HCO₃⁻ | 13.2 | 10.1 | 15.2 | 12.83±2.5 | 11.8 | 14.2 | 11.4 | 12.4±1.5 |
| Tot Hb | 10.8 | 11.2 | 10.2 | 10.73±0.5 | 8.5 | 10.0 | 10.7 | 9.7±1.1 |
| % Htc  | 36.0 | 37.4 | 34.0 | 35.8±1.7 | 29.3 | 33.2 | 35.7 | 32.7±3.2 |
growth. For instance, it was estimated that osmoregulation might consume as much as 54-68% of the non-swimming metabolic output in two species of tunas (Bushnell and Brill, 1992). Even in species with lower metabolic rates, osmoregulation appears to use a high proportion of the available energy (Nilsson, 2007), while Boeuf and Pajan (2001) reported that it ranges from 20 to 50% of the total energy expenditure, roughly 100-150 mL h⁻¹ kg⁻¹ O₂ depending on the environmental salinity. Theoretically, a gill ideally designed to absorb oxygen for the energy requirements should be highly permeable, with quite a large surface area, leading to an ascending metabolic cost of osmoregulation (Grau et al., 1994). These conflicting demands may represent the major constraint in the overall metabolic capacity of teleostean fishes. All fishes appear to be limited by the need for an ‘osmo-respiratory compromise’ (Nilsson, 1986). The energy cost of ionic regulation increases when the salinity is altered away from iso-osmotic conditions, but any attempt to quantify this cost is probably affected by other metabolic processes that respond to changes in salinity (Morgan and Iwama, 1991).

A central process in osmotic regulation is active Na⁺ transport; a great deal of energy is consumed in moving ions through a Na⁺-K⁺-ATPase, but rather via the basolateral pathway, either through diffusion or an active bulk flow mechanism; thus, Boeuf and Pajan (2001) argued that the energy required could be underestimated. Many of the ions transported by ATPases may simply diffuse back out of the basolateral network into body fluids or, if the bulk flow hypothesis is correct, the energy needed to drive it would also have to be estimated. Another element reported by the latter authors is that accepting a low cost for osmoregulation mechanisms would contradict the demonstrated effect of water salinity, especially in the case of saltwater, on the growth of teleosts (Insland et al., 2001).

Evidence exists that elevate PO₂ regimen allow fish to save energy (Saroglia et al., 1998b). Moreover, an ‘on farm’ observed improvement in quality (Iandoli et al., 1995), increased immunoglobulbon concentration (Scapigliati et al., 1999), and specific antibody response (Saroglia et al., 1998a), when pure oxygen is applied instead of simple aeration, sound compatible with an energy saving for osmoregulation.

Conclusions

Our data are compatible with the hypothesis of an osmotic advantage of sea bass exposed to an elevated PO₂ regimen. With respect to previously reported literature information on the effect of PO₂ on fish GDD, FSA and RSA, this could tentatively be compatible with an advantage for osmo-respiratory compromise as well. Such results would imply that a consistent energy advantage would significantly reduce the cost on fish metabolism. Therefore, a correct evaluation of the cost for osmoregulation is envisaged and the question of the actual energy savings provided by elevated PO₂ in water is still open.

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