Influence of CO₂-induced seawater acidification on the development and lifetime reproduction of *Tigriopus japonicus* Mori, 1938

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Ocean acidification is changing the carbonate system of the world's oceans and has been driving all marine organisms to live in increasingly acidic environments. *Tigriopus japonicus* is an ideal standard test animal in sea water. In the present study, we investigated the influence of carbon dioxide (CO₂)-induced seawater acidification on the development and lifetime reproduction of *T. japonicus* to accumulate basic data for assessing the potential impact of ocean acidification. The harpacticoid copepods were exposed in seawater equilibrated with CO₂ and air to reach pH 8.0 (control), 7.7 (the predicted ocean pH by 2100), 7.3 (the predicted ocean pH by 2300) and 6.5 (an extreme condition relevant to industrial acid waste discharge or leakage from CO₂ seabed storage). Survival was found to be unaffected following the 56 day exposure period. Significant retardation of development rates of the nauplius stage was observed at pH 6.5, while the development time of the copepodite stage was unaffected. Acidification did not affect the number of broods but it significantly reduced the hatching success of egg sacs at pH 6.5. Total production of nauplii over the lifetime of female copepods was significantly reduced at pH 7.3. Over successive broods, nauplius production was significantly affected by exposure time, pH and their interaction. Shedding of unhatched egg sacs by females mainly occurred in the late breeding stage at pH 7.3 and 6.5. Our results indicated that *T. japonicus* adults are tolerant to the ocean acidification conditions predicted for the year 2100, but the early development and reproductive capacity of females could be impaired by long-term exposure to more severe acidification conditions (pH 7.3 and 6.5). More long-term studies on a wider range of copepod species from different taxa and different marine habitats are urgently required to predict the fate of marine copepod communities in future oceans.

Keywords: ocean acidification; development; reproduction; harpacticoid; *Tigriopus japonicus*

Introduction

Ocean acidification is causing worldwide concern and it is gaining recognition alongside climate change as the other global carbon dioxide (CO₂) problem (Doney et al. 2009). More than a third of the CO₂ emitted into the atmosphere since the beginning of the industrial revolution has been absorbed by the oceans, resulting in an alteration in the seawater carbonate system to increasing the hydrogen ion (H⁺) concentrations and concurrently reducing the carbonate ion concentrations (Feely et al. 2004). Ocean surface water pH has fallen by 0.1 units since pre-industrial times, and it is predicted that it could fall by up to 0.3~0.4 units before 2100 (Orr et al. 2005; IPCC 2014). A reduction of 0.7 units could possibly occur by 2300 (Caldeira and

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Wickett 2003). Leakage from CO₂ seabed storage would create locally stronger acidification than that induced by atmospheric CO₂ (Barry et al. 2004).

The biological effects of ocean acidification are still far from clear (Whiteley 2011; Cripps et al. 2014). Calcifying marine organisms such as echinoderms, molluscs and corals have received the most attention because they are believed to be particularly sensitive to elevated CO₂ levels (Widdicombe and Spicer 2008; Byrne 2011). However, our limited understanding of the effect of ocean acidification on other marine taxa is a knowledge gap. Copepods are the most abundant metazoa (Boxshall and Halsey 2004) and are ecologically important as they are primary and secondary consumers and an important food source for higher trophic levels in oceans (Runge 1988). There are an increasing number of studies investigating the effect of ocean acidification on marine copepods. However, most previous studies only cover part of the life cycle of many species (such as Kurihara et al. 2004; Mayor et al. 2007, 2012; Pascal et al. 2010; Zhang et al. 2011, 2012; Li and Gao 2012; Weydmann et al. 2012; McConville et al. 2013; Pedersen et al. 2013; Hildebrandt et al. 2014), and only a few have included the full life cycle or multiple generations (Kurihara and Ishimatsu 2008; Fitzer et al. 2012, 2013; Kita et al. 2013; Pedersen et al. 2014). At the same time, the published studies that have examined long-term impacts of ocean acidification on copepods mainly concentrate on pelagic copepods and have included only a few reports on benthic copepods (Fitzer et al. 2012; Kita et al. 2013). Harpacticoids are an important component of the marine benthic ecosystem, inhabiting diverse benthic habitats and utilising different survival strategies compared to pelagic copepods (Hicks and Coull 1983). Although marine copepods are perceived to be relatively resilient to ocean acidification when compared to other organisms (Whiteley 2011), the tolerances of copepods from different habitats (pelagic versus benthic, pH-stable versus pH variable) might differ.

Tigriopus japonicus Mori, 1938 is a harpacticoid copepod species which is commonly found in tidal pools in the upper intertidal zone of the western Pacific Ocean. Previous studies have demonstrated that it represents a suitable model species for acute and chronic assessment of marine pollutants (Lee et al. 2008). As a test species it has several advantages, including distinct sexual dimorphism, distinct nauplius and copepodite stages, high fecundity, a short life cycle and ease of culture (Raisuddin et al. 2007). These properties have enabled us to conduct an investigation of the species' response to CO₂-induced acidification over its total lifespan. The range of natural variability in the partial pressure of carbon dioxide ($p\text{CO}_2$) that copepods experience may determine their sensitivity to future ocean acidification (Lewis et al. 2013). *Tigriopus* is generally tolerant of drastic environmental fluctuations in tidal pools (Davenport et al. 1997), and therefore it may be expected to be less sensitive to future ocean acidification than other copepod species from more stable environments. However, having the ability to endure these short-term environmental fluctuations could make copepods pre-adapted to survive long-term exposure to ocean acidification. To our knowledge, only one previous study has used *T. japonicus* as the test organism to study the long-term effects of ocean acidification, documenting developmental delays of the nauplius phase and decreased hatching success of the first spawned egg at pH 6.31 ($p\text{CO}_2$: 37,000 μatm ; Kita et al. 2013). Sensitive life phases, such as early developmental stages and reproductive periods, represent bottlenecks for the population; as such, these stages could be more vulnerable to the effects of ocean acidification. Any factor that influences the growth and

number of progeny has the potential to drive change at the population scale and affect species survival, distribution and abundance (Dupont and Thorndyke 2009; Halsband and Kurihara 2013). The effects of ocean acidification on different life stages and the lifetime reproduction of *T. japonicus* are still unknown. The aim of this study was to assess the sensitivity of *T. japonicus* to long-term exposure to ocean acidification conditions. We exposed the animals from the earliest nauplius stage up to adults under controlled laboratory conditions and determined survival rate, development rate of the nauplii and copepodite stages, and lifetime fecundity.

Materials and methods

Test animals

The harpacticoid copepod *Tigriopus japonicus* was collected from a high-tidal rock pool at Huiquan Bay, Qingdao, China. A stock culture was maintained in 0.45- μ m micro-filtered natural seawater (30 salinity, renewed every 2 weeks) at $20 \pm 1^\circ\text{C}$ under a 16:8hr light: dark photoperiod in an incubator. Copepods were fed weekly with *Phaeodactylum tricornutum* Bohlin and commercially available baker's yeast (*Saccharomyces cerevisiae*; Anqi®, China) in equal proportions (10×10^4 cells/mL in total). Our preliminary feeding study showed that *T. japonicus* developed and reproduced very well when fed a mixture of *P. tricornutum* and yeast, the optimal food concentration being from 5×10^4 to 30×10^4 cells/mL. To avoid the influence of metabolism and decomposition of excess food, a food concentration at the lower end of this range was used in the experiments.

Treatment levels

The life stages of *T. japonicus* were exposed to four different pH levels induced by CO₂ enrichment: (1) pH 8.0, the ambient seawater pH measured at the collection site, which was used as the control; (2) pH 7.7, 0.3 units below ambient, the predicted drop in ocean pH by 2100 (Orr et al. 2005; IPCC 2014); (3) pH 7.3, 0.7 units below ambient, the predicted drop in ocean pH by 2300 (Caldeira and Wickett 2003); (4) pH 6.5, an extreme level related to industrial acid waste discharge or leakage from CO₂ seabed storage (Barry et al. 2004). The CO₂-rich seawater (pH 4.5) was prepared by bubbling pure CO₂ (99.99%) into 0.45- μ m micro-filtered natural seawater. The experimental levels of seawater pH were obtained by mixing different proportions of this high-*p*CO₂ seawater with seawater saturated with ambient CO₂ levels. Measurements of pH (NBS pH scale) were made using a pH meter (Mettler-Toledo®, DELTA 320, Switzerland) with a combination ATC probe. The pH meter was calibrated with buffer solutions of pH 4.01, 6.86 and 9.18 (Mettler-Toledo®) before use. Dissolved oxygen was measured using a dissolved oxygen meter (Leici®, JPB-608, China) calibrated using distilled water as 100% oxygen (O₂) reference and 5% sodium sulphite (Na₂SO₃) solution as an oxygen-free standard. The seawater salinity (PSU) was measured with a refractometer (Zhengzhou Nanbei Instrument Equipment Co. Ltd., HB-212ATC, China). The total alkalinity of seawater was measured by the hydrochloric acid titration method (Shi et al. 2008). The salinity and total alkalinity were measured at the beginning of the experiment. The partial pressure of CO₂, the total dissolved inorganic carbon and the calcium

carbonate saturation state for calcite (Ω_{Ca}) were calculated using the computer program SWCO2 (http://neon.otago.ac.nz/research/mfc/people/keith_hunter/software/software.htm), from the pH and total alkalinity. Seawater properties were determined using CO_2 equilibrium constants given by Mehrbach et al. (1973), revised by Dickson and Millero (1987).

Development and reproduction experiment

For both experiments, glass bottles with a 10-mL volume were washed in deionised water and filled with seawater at the required pH. After transferring test animals and food to the bottles, they were sealed with airtight rubber lids, then further sealed with aluminium caps (using a capping machine) to ensure there was no exchange of gases with the environment. The experiments were conducted at $20 \pm 1^\circ\text{C}$ under a 16:8-hr light:dark photoperiod in a climate chamber (Ningbo Jiangnan Instrument Co. Ltd., GXZ, China). *P. tricornutum* and yeast in equal proportions were added to the rearing seawater as food. Dissolved oxygen and pH were monitored at the time of renewal of the test solutions.

Females with eyed eggs were collected from the cultivation stocks. Twenty newly hatched nauplii (< 24 hr after hatching) were transferred to each of three replicate bottles for each pH level. The nauplii were cultured until they reached adulthood and females developed egg sacs. The development stage was observed daily under a stereomicroscope (Jiangnan®, SE2200, China) and recorded to calculate the time of nauplius stage (days needed until 50% of nauplii moulted to copepodites) and copepodite stage (days needed until 50% of copepodites moulted to adults). The survival rate (%) was determined after the maturation of all copepods. In the development experiments, the *P. tricornutum* and yeast were added at a final density of 10×10^4 cell/mL. The test solution in each bottle was renewed every second day.

Two females bearing egg sacs from each bottle in the development experiment were picked and individually transferred to a new glass bottle for each of the three replicates at each treatment level. These females were cultured until they stopped producing egg sacs, or died before the experiment was terminated at 56 days after the start of the experiment. The total number of broods, the number of viable egg sacs and the number of nauplii per viable egg sac were recorded during the 56 days. In the reproduction experiment, food was added at a final density of 5×10^4 cell/mL. The test solution was renewed daily when the resulting nauplii and the fallen egg sacs were counted under the stereomicroscope and removed.

Statistical data processing

Effects of acidification on development and reproduction parameters were evaluated statistically using SPSS (v. 17.0). Levene's tests for homogeneity of variance were used to assess the normality of the data. One-way analysis of variance (ANOVA) or Kruskal–Wallis tests (for non-parametric data) were carried out to determine whether the parameters were significantly affected by seawater acidity. Tukey honestly significant difference (HSD) multiple comparisons or independent t-tests were used in comparisons of treatments and control. Two-way ANOVA was used to determine if nauplius reproduction was significantly affected by the combined effect of pH and exposure time (represented by sequence of brood). All statistical analyses were tested

for significance at the 5% level (significant, $p < 0.05$). Values were given as means $\pm$ standard deviation (SD).

Results

Environmental monitoring

The measured and the calculated values for the water parameters are presented in Table 1. Dissolved oxygen concentration at pH 7.3 and 6.5 was lower than at pH 7.7 and 8.0. Monitoring of pH and dissolved oxygen indicated that these parameters remained stable throughout the experiment.

Effect of acidification on development

The development time of the nauplius phase was significantly affected by acidification (Figure 1) according to the Kruskal–Wallis test ($H = 9.914$, $df = 3$, $p < 0.05$). Acidification showed no significant effect on the development time of the copepodite phase ($H = 6.600$, $df = 3$, $p > 0.05$). Development time of the nauplius phase showed a significant delay at pH 6.5 compared to pH 8.0 (t-test: $t = -5.000$, $df = 2.000$, $p < 0.05$).

Effect of acidification on survival rate of larvae

The survival rate of larvae (the percentage of the nauplius stage that reached the adult stage) showed no significant difference among four pH levels (one-way ANOVA: $F_{(3, 8)} = 2.617$, $p > 0.05$).

Effect of acidification on reproduction

Production of egg sacs and hatching success

In the reproduction experiment, no female mortality was observed across all treatments, including controls. Acidification did not affect the number of broods

Table 1. Environmental parameters in control and treatments. pH (NBS scale), temperature (T), salinity (S), dissolved oxygen (DO) and total alkalinity (A_T) were measured. All other values (partial pressure of carbon dioxide: pCO_2 ; total dissolved inorganic carbon: C_T ; calcium carbonate saturation state for calcite: Ω_{Ca}) were calculated using SWCO2 from pH and total alkalinity. The values of pH and dissolved oxygen represent means [$\pm$ standard deviation (SD); $N = 28$] over the course of the experiment. The other values indicate means ($N = 3$) at the beginning of the experiment.

	Target pH	Actual pH _{NBS}	S (PSU)	T (°C)	DO (mg/L)	A_T (mmol/L)	pCO_2 (μ atm)	C_T (μ mol/L)	Ω_{Ca}
Control	8.0	7.99 ($\pm$ 0.02)	29.0	20	7.52 ($\pm$ 0.13)	2.024	611	1898	2.48
Treatment 1	7.7	7.74 ($\pm$ 0.02)	29.1	20	7.59 ($\pm$ 0.09)	2.075	1176	2026	1.52
Treatment 2	7.3	7.34 ($\pm$ 0.02)	29.0	20	6.76 ($\pm$ 0.15)	2.003	2983	2067	0.61
Treatment 3	6.5	6.57 ($\pm$ 0.03)	29.4	20	6.81 ($\pm$ 0.07)	2.038	18269	2640	0.11

Figure 1. Effect of carbon dioxide (CO_2)-induced seawater acidification on development time [mean $\pm$ standard deviation (SD), $N = 3$] of *Tigriopus japonicus* (N-C, nauplius to copepodite; C-A, copepodite to adult).

Figure 2. Effect of carbon dioxide (CO_2)-induced seawater acidification on the number of broods ($N = 3$) produced by females of *Tigriopus japonicus* over the duration of the experiment (median indicated with a bar; quartiles, minimum and maximum also shown).

Figure 3. Proportion of egg sacs that successfully produced nauplii ($N = 3$) at four pH levels (median indicated with a bar; quartiles, minimum and maximum also shown).

(Figure 2; Kruskal–Wallis test: $H = 4.051$, $df = 3$, $p > 0.05$), but it significantly reduced the proportion of viable egg sacs (Figure 3; one-way ANOVA: $F_{(3, 8)} = 25.788$, $p < 0.05$). Shedding of unhatched egg sacs (which became green in colour) became very evident at low pH, with the proportion of viable egg sacs decreasing from 91% and 92% at pH 8.0 and 7.7 to 84% (pH 7.3) and 57% (pH 6.5). The pairwise test confirmed the significant difference in hatching success at pH 6.5 compared to pH 8.0 (Tukey HSD: $p < 0.05$).

Lifetime output of nauplii by females

The total production of nauplii over the duration of the experiment was significantly decreased by CO_2 -driven seawater acidification (Figure 4; Kruskal–Wallis test: $H = 8.436$, $df = 3$, $p < 0.05$). The t-test showed a significant decrease at pH 7.3 compared to pH 8.0 ($t = 4.794$, $df = 2.133$, $p < 0.05$). The average number of nauplii per viable egg sac was not affected by acidification (Figure 5; one-way ANOVA: $F_{(3, 8)} = 1.655$, $p > 0.05$).

Figure 4. Effect of carbon dioxide (CO₂)-driven seawater acidification on total number of nauplii (N = 3) produced by *Tigriopus japonicus* females over the duration of the experiment (median indicated with a bar; quartiles, minimum and maximum shown).

Variation in nauplius production with pH and exposure time

Two-way ANOVA tests showed that nauplius production was significantly affected by the main effects of sequence of brood ($F_{(11, 96)} = 5.808, p < 0.05$), pH level ($F_{(3, 96)} = 3.270, p < 0.05$) and their interaction ($F_{(33, 96)} = 1.750, p < 0.05$). Tukey HSD showed a significant reduction in nauplius production at pH 6.5 and pH 7.3 compared to pH 8.0 ($p < 0.05$). The production of nauplii fluctuated during the breeding period. There appeared to be two production peaks near the third and the eighth brood at all pH levels. However, after the second peak, the production at pH 7.3 and 6.5 fell more rapidly than at the other two pH levels, and plummeted to zero at pH 6.5 (Figure 6). These differences appeared to be caused principally by an increase in the number of egg sacs which failed to develop (i.e. dropped off) rather than a reduction in brood size at the later stages of the breeding cycle at lower pH levels. Indeed, at pH 6.5 all the female copepods discarded their egg sacs during the final two broods.

Figure 5. Average number of nauplii per viable egg sac ($N = 3$) at four pH levels (median indicated with a bar; quartiles, minimum and maximum also shown).

Discussion

Dissolved oxygen concentration of the test solution in an enclosed bottle was closely linked with respiration and photosynthesis of the test animals and food organisms. In this experiment, dissolved oxygen at pH 7.3 and 6.5 was lower than at pH 7.7 and 8.0 (Table 1), which may have indicated that there was a higher respiration rate of copepods and/or a lower photosynthesis rate of algae under lower pH conditions. The saturation concentration of seawater is 7.6 mg/L at a temperature of 20°C and salinity of 29 PSU (GB/T12763-2007 2007). Dissolved oxygen concentrations in the four experimental treatments were close to the saturation concentration. A 10% reduction (less than 0.8 mg/L lower in the treatments compared to the control) probably had little effect on the results of this experiment.

Retarded larval development under high- CO_2 conditions has been observed in a wide variety of marine taxa, and most evidence indicates that ocean acidification is a major threat to calcifying larvae, because it decreases availability of the carbonate ions required for skeletogenesis and also exerts a direct pH effect on physiology (Dupont and Thorndyke 2009; Byrne 2011). The responses of copepod larvae to ocean acidification are relatively poorly understood, and research results are inconsistent. Kurihara and Ishimatsu (2008) showed that development of the calanoid

Figure 6. Variation in number of nauplii [mean $\pm$ standard deviation (SD), $N = 3$] produced by *Tigriopus japonicus* females over successive broods at four pH levels.

copepod *Acartia tsuensis* was unaffected by CO_2 treatment (pH 7.32, equivalent to a CO_2 concentration of 2380 ppm in their experimental system) at all stages. For the calanoid copepod *Calanus finmarchicus*, Pedersen et al. (2014) observed a significant delay in development rates among the parental generation for animals exposed to 2080 μatm CO_2 , but not in the following F1 generation under the same conditions. In another study, Cripps et al. (2014) found that nauplii of the calanoid copepod *Acartia tonsa* showed sublethal retarded growth for the ‘near-future’ level of $p\text{CO}_2$ (pH 7.84, equivalent to 1000 μatm $p\text{CO}_2$ in their system). Kita et al. (2013) found that the time needed for first appearance of copepodites of *T. japonicus* was prolonged at pH 6.31, but not at 7.11 and above. The findings in the present study, using the same test animal, are consistent as we also demonstrated a significant delay of the nauplius stage under highly acidified conditions (pH 6.5), but also showed that the development time of the copepodite stage was not affected. The retarded development rate observed in the nauplius stage at pH 6.5 could result from energy cost balance against the increased acidity and CO_2 concentration. Pedersen et al. (2014) reported elevated metabolism in *C. finmarchicus* that had experienced long-term CO_2 exposure. Despite increased metabolic costs, no increase in feeding was observed, resulting in reduced scope for growth among the CO_2 -exposed copepods. Their results indicated that negative effects observed on vital rates (ontogenetic development, somatic growth, fecundity) may be a consequence of energy budget constraints due to higher maintenance costs in high $p\text{CO}_2$ -environments. Calcification processes in crustaceans are likely to be less vulnerable to ocean acidification because exoskeletal calcium carbonate (CaCO_3) is mostly in the more stable form of calcite rather than the more soluble

aragonite (Neues et al. 2007). High organic (chitin, protein) content of cuticle also could make the crustaceans more resilient to ocean acidification (Byrne 2011). Fitzer et al. (2012) found there was a significant increase in the proportion of carbon relative to oxygen in the cuticle as seawater pH decreased. However, the cuticle of copepods contains very little calcium carbonate: copepods possess a chitinous exoskeleton rather than an aragonite or calcite shell, and should be considered to be non-calcified (Fitzer et al. 2012).

The present study showed that total nauplius output over the female lifetime was significantly reduced at pH 7.3. These results are in agreement with many previous observations (e.g. Kurihara et al. 2004; Mayor et al. 2007, 2012; Weydmann et al. 2012; McConville et al. 2013; Hildebrandt et al. 2014), suggesting that CO₂-induced seawater acidification can negatively affect reproduction in copepods, especially in some cases of long-term and multi-generation exposure. Kurihara and Ishimatsu (2008) studied the effects of exposure to acidic seawater (CO₂ 2380 ppm) on the copepod *A. tsuensis* from eggs to maturity and over two subsequent generations. The results revealed that there was a significant difference in the hatching rate between control and CO₂ groups, although there was no difference when compared separately for each generation. Incubated for three generations, the growth and reproductive rate of the harpacticoid copepod *Tisbe battagliai* decreased at pH 7.82 (Fitzer et al. 2012). The present study found more pronounced detachment of unhatched green egg sacs in the late period of the experiment at low pH levels (pH 7.3 and 6.5). Kita et al. (2013) observed transparent and green undeveloped eggs at a *p*CO₂ level of 37,000 µatm (equivalent to pH 6.31 in their system). Zhang et al. (2012) reported for two calanoids (*Calanus sinicus* and *Acartia spinicauda*) that the mitochondria were damaged below pH 7.34 and that spherical granules were concentrated or collapsed. This evidence indicated that CO₂-driven acidification could induce apoptosis and impact the quality of copepod eggs, with potential further damage as exposure times increase. Long-term exposure might cause cumulative detrimental effects and impair the reproductive capacity of copepods.

Within this study, the survival rate of larvae in the CO₂ treatments was not significantly different from the control, and no adult female mortality was observed at any of the pH levels over the duration of the reproduction experiment. In addition, both the developmental and reproductive parameters were only significantly different from the controls at pH 7.3 and pH 6.5, demonstrating that *T. japonicus* will be able to tolerate the levels of ocean acidification predicted for the rest of this century. On short time scales, these copepods are used to a fluctuating environment. Almén et al. (2014) reported that pelagic copepods experienced a change in pH of more than 0.5 units (larger than the predicted near-future ocean acidification) during diel migration. *Tigriopus japonicus* can tolerate drastic variations in the physicochemical environment of high-tidal rock pools (Morris and Taylor 1983). The diurnal variation of pH in tidal pools ranged from 7.20 to 9.50 (Daniel and Boyden 1975). Although *T. japonicus* seems to be one of the most robust copepod species, a late effect on female reproduction was observed in this study at pH 7.3 and 6.5, which indicates that *T. japonicus* may not be as tolerant to long-term exposure to CO₂-induced acidification as previously assumed. At the same time, although acidification is changing the oceans gradually, it is still a matter of conjecture whether marine copepods could adapt and outpace the rate of change. Once acidification reaches the species-specific *p*CO₂

tolerance limit, any additional stressor (be it anthropogenic or natural) could adversely affect the response of the individual to this climate change stressor. Pedersen et al. (2014) observed a retarded development rate among parental-generation copepods raised at 2080 ppm, but normalisation of the development rate among their F1-generation offspring when raised under the same conditions, suggesting that copepods could have the potential to adapt to future ocean acidification conditions. More long-term (preferably multi-generation) studies of the effects of ocean acidification on full life-cycle traits and potential adaptation are urgently required, and a wider range of copepod species from different taxa and different marine habitats should be examined to predict the fate of marine copepod communities in future oceans.

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