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Pattern of secondary infection with *Saprolegnia* spp. in wild spawners of UDN-affected sea trout *Salmo trutta* m. *trutta* (L.), the Słupia River, N Poland

by

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Abstract

Ulcerative dermal necrosis (UDN), a disease of unknown etiology affecting salmonids, has been observed in some rivers in the north of Poland since 2007. Fish affected by UDN die, inter alia, as a result of secondary infection caused by water molds from the *Saprolegnia* genus. UDN is considered to be an important factor affecting the decline of Polish salmonid populations observed in recent years. Patterns of secondary infection and differences between sexes in UDN-affected wild, adult sea trout (*Salmo trutta* m. *trutta*) during the 2014 and 2015 spawning season have been described on the basis of photographic documentation and image analysis. Male fish are most commonly infected in the dorsal region, while females in the head and tail areas. When comparing the infection patterns in both sexes, it is clear that large areas of flanks are significantly more frequently infected in males than in females. The dorsal half of the tail fin is most commonly affected by pathological changes in females. These findings are discussed in relation to various factors predisposing salmonids to *Saprolegnia* spp. infections during the spawning season. The presented infection patterns can be helpful in preliminary diagnosis of early stages of secondary infection with *Saprolegnia* spp. in UDN-affected salmonids.

Key words: ulcerative dermal necrosis, *Saprolegnia*, sea trout, secondary infection, infection pattern

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Introduction

Sea trout – *Salmo trutta* m. *trutta* (L.) is a form of brown trout – *Salmo trutta* (L.) – capable of anadromous migration (Bouza et al. 1999). Fish occurring in Polish coastal waters are of Atlantic origin (Bernatchez 2001). Despite a decrease in landings in recent years, sea trout is still the most common salmonid fish in Polish commercial fishing (139 tons in 2016) (Central Statistical Office of Poland 2017). Anadromous fish are considered a "keystone" species in aquatic and terrestrial environments. They affect many other organisms in the ecosystem, and therefore play an important role in the ecological community (Willson & Halupka 1995).

In Poland, sea trout migrates for spawning mainly into coastal rivers and streams of Pomerania (northern Poland) (Dębowski & Bartel 1995). After hatching from eggs, fry live in freshwater for up to two years. Then they turn into smolts, which migrate to the open sea (Dolina Słupi Landscape Park 2017). When trout reach sexual maturity (after another 1–2 years), they return to their birthplace for spawning. During this event, fish can lay eggs several times. Migrations occur in September and November, and spawning in November (Was & Wenne 2002).

The conservation status of sea trout is described as Least Concern (LC), but HELCOM proposes to treat Baltic populations of anadromous fish as Vulnerable (VU) (Froese & Pauly 2017; HELCOM 2013).

The main causes of being threatened are migration barriers and overfishing. Small hydropower stations limit the access of fish to spawning grounds, altering the river water levels during spawning and changing natural habitat conditions (Radtke et al. 2012). Excessive commercial fishing, angling and poaching contribute greatly to the decline of sea trout populations in Poland (HELCOM 2015). In addition, environmental changes related to water pollution, drainage and regulation of rivers and streams are of great concern (Dolina Słupi Landscape Park 2017).

One of the factors limiting the sea trout populations is Ulcerative Dermal Necrosis (UDN). It is a skin condition of unknown etiology affecting adult, wild salmonid fish migrating from open seas to freshwater streams for spawning (Bruno et al. 2013; Harris et al. 2011). First mentions of a similar disease were reported at the end of the 19th century in Great Britain (Bruno et al. 2011; Roberts 1993). The first UDN outbreak in modern times occurred in southwestern Ireland in 1964 (Munro 1970). Since then, it has been observed in Great Britain, France (Roberts 1993), Sweden (Johansson et al. 1982), Austria, Belgium, Luxembourg, Germany, Switzerland and Canada (Grudniewska et al. 2012).

The disease remains in rivers for 7–8 years with heaviest mortalities in the first 3–4 years (Roberts 1993). It occurs from late autumn to spring, with the highest mortalities in November and December, when the water temperature drops. This is also the sea trout mating season. Large concentrations of fish provide excellent conditions for the spread of contagious diseases (Munro 1970). There is evidence that UDN can be water-transmitted (Johansson et al. 1982; O'Brien 1974, personal observations).

The first UDN symptoms are small greyish lesions located on the operculum and scaleless skin of the head. This is a sign of progressive cytolytic necrosis of epidermal Malpighian cells. When reaching freshwater, lesions rapidly ulcerate and often become infected with *Saprolegnia* spp. water mold, the growth of which causes an increase in lesions (Bruno et al. 2013; Roberts 1993). *Saprolegnia parasitica* is a fungus-like protist belonging to the *Oomycota* class. These organisms are known to be brackish and freshwater fish pathogens. Their resemblance to fungi is misleading, as in fact they are closely related to golden-brown algae (*Chrysophyceae*) (Roberts 2012). The order of Saprolegniales includes three of the best-studied water mold genera: *Saprolegnia*, *Achyla* and *Aphanomyces*. Two closely related species: *S. diclina* and *S. parasitica*, found in temperate climate areas, have significant impact on salmonid aquaculture. Saprolegniosis caused by these oomycetes affects various fish species. *S. parasitica* is mostly linked to fish infections in aquacultures. Usually patches of the cotton wool-like mycelium are located on the host's skin or gills, but they can also be found on eggs. It is most visible when fish is immersed in water. In some cases, up to 80% of the skin can be covered with mycelia (Van Den Berg et al. 2013). Similar coverage of the hyphae was observed in trout from the Słupia River in 2014 and 2015 (personal observations). Initial lesions are often circular, small and superficial, but in some cases mycelia extend into the dermis and adjacent muscles (Bruno et al. 2013; Khoo 2000). Oomycete infections lead to histological changes – loss of epidermis integrity, edema and degeneration of superficial muscle tissue. Severe lesions are characterized by deeper cellular necrosis, spongiosis and epidermis cell sloughing (Bruno et al. 2011; Copland & Willoughby 1982). Lethargic behavior is the result of epidermis and underlying tissue damage. Final infection stages are mainly indicated by respiratory failure and impaired osmoregulation (Bruno et al. 2011; Pickering & Willoughby 1982). Respiratory failure is caused by gill damage. Impaired osmoregulation is a result of hemodilution induced by considerable epidermis

damage (Van Den Berg et al. 2013). Death is caused by secondary bacterial infections or, in most cases, circulatory failure following the osmotic hemodilution (Roberts 1993). First mortalities occur 3–4 days after the appearance of the first visible lesions (personal observations).

The first documented report of a UDN-like disease in Poland describes characteristic lesions and mortalities among Atlantic salmon, *Salmo salar* (L.) from the Dunajec River (Lubecki & Dixon 1925). Since then, there were no mentions of a similar disease in Poland until 2007, when UDN was observed in rivers of northern Poland (Bartel et al. 2009; Grudniewska et al. 2011; 2012; Kazuń et al. 2011). One of the first rivers affected by the disease was the Słupia River, running through the Dolina Słupi Landscape Park. Since 2007, sea trout specimens with characteristic UDN ulcers have been observed on a yearly basis. Based on early clinical signs, furunculosis was suspected as the main disease. It is a highly contagious disease caused by *Aeromonas salmonicida* (Bartel et al. 2009). This Gram-negative bacteria is an opportunistic pathogen known to cause heavy mortality and considerable economic loss in aquacultures (Hu et al. 2012; Kozińska 2007; Wiklund & Dalsgaard 1998). The main furunculosis symptoms are: lesions, anorexia, exophthalmus, skin hemorrhage, apathy and weakness (Hu et al. 2012). Despite considerable efforts, *A. salmonicida* was not found in diseased animals, so furunculosis was ruled out. Clinical signs indicated UDN (Grudniewska et al. 2011; 2012; Kazuń et al. 2011). Further microbiological assessment of UDN-affected sea trout from small rivers in northern Poland (including the Słupia River) conducted in 2010 showed that *Saprolegnia* spp. water molds are present in damaged skin of these fish alongside with bacteria and yeasts (Kazuń et al. 2011).

The objective of this paper is to describe patterns and distribution of secondary *Saprolegnia* spp. infections on the body of mature, wild, UDN-affected sea trout, *Salmo trutta* m. *trutta* from the Słupia River in the 2014 and 2015 spawning seasons, and their relation to sex and gender biology.

Based on field observations, we assume that there are differences in secondary infection patterns depending on the body surface area and sex. These differences may be caused by disparity in gender biology.

Materials and methods

Fish

Mature, UDN-affected wild spawning sea trout (*Salmo trutta trutta*) were caught in November 2014 and 2015 in the Słupia River, Słupsk, northern Poland. Water temperature during observations ranged from 4.6 to 8.6°C. All specimens were caught at a Polish Angling Association trapping point (54°27'37.4"N, 17°02'21.1"E) during annual artificial trout spawning. In 2014 and 2015 together, 106 (44 male and 62 female) specimens were caught. All animals with visible signs of infection, used in this study, were bycatch from catches targeting healthy specimens required for artificial spawning and were euthanized by means of manually applied blunt force trauma to the head followed by pithing. Fish for this research were provided by the Polish Angling Association. Trout were then sexed, weighed and their length was determined. Photographic documentation of infected spots on the fish body covered both sides of each specimen, and the infected area close-ups were taken with a Nikon D80 camera.

Image analysis and statistics

Schemes of oomycete infection (Fig. 1) were prepared on the basis of photographic documentation for each specimen in GIMP 2.8.18 software (the GIMP

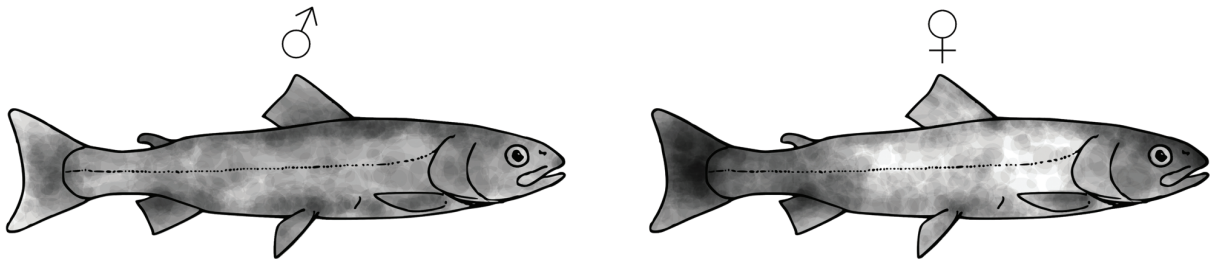

Figure 1

Comparison of composite schemes of *Saprolegnia* spp. infected males and females of UDN-affected sea trout. The darker the area, the higher the frequency of secondary infection.

team, www.gimp.org, 1997–2016). All visible signs of infection were mapped on an outline representing a trout silhouette. Two schemes were prepared for each specimen (for left and right sides of the body). Due to visible bilateral symmetry of lesions, schemes prepared for each side of the body were merged together for the purpose of analysis. Böhmig & Brauer (1909) *Salmo trutta* m. *fario* illustration was chosen due to its simplicity and similarity of morphological aspects to those of *S. trutta* m. *trutta*. These features make the infection scheme comparisons easier and more unequivocal. For the same reasons, one outline was used for both sexes.

To determine the most commonly oomycete-affected spots on the fish body, all individual schemes for each sex were merged together (Fig. 1). Places with the highest oomycete occurrence were marked as the darkest. Each composite image was then divided into 298 squares. ImageJ 1.50i (Rasband 1997) image processing and analysis software was used at this point. Square mean color intensity analysis was conducted for each composite image. The number of infected specimens for each square in a given image was determined based on the obtained data. Incidence of infection for each square was calculated (the number of infected specimens in a square divided by the number of specimens in a sample). A Chi-square test was used to compare the calculated incidence for each square with mean incidence for all squares:

$$\chi^2 = (INF_{obs} - INF_{exp})^2/INF_{exp} + (NotINF_{obs} - NotINF_{exp})^2/ NotINF_{exp}$$

INF_{obs} – the number of specimens with the identified infection in a given square

INF_{exp} – the mean number of infected specimens for all squares, used as an expected value

$NotINF_{obs}$ – the number of specimens in which the infection was not identified in a given square

$NotINF_{exp}$ – the mean number of uninfected specimens for all squares, used as an expected value

On this basis, we were able to determine significantly more infected and uninfected areas, separately for male and female fish. The results of the analysis were then replotted on a sea trout outline (Fig. 2).

To compare infection patterns between sexes, schemes for each individual specimen were divided into 298 squares, and then analyzed separately with ImageJ 1.50i (Rasband 1997). At this point, the presence or absence of pathological changes in each square for each specimen was obtained. Results for males and females were compared by Chi-square 2 × 2 contingency tables with the use of the Statistica 12.5 (StatSoft 2006) software. The results of the analysis were then replotted on a sea trout outline.

Results

When comparing composite images prepared for both sexes, the difference in the distribution of infection between male and female fish is clearly visible (Fig. 1). In males, the most frequently infected body areas are the dorsal body surface with the anterior end of the dorsal fin and the adipose fin. In females, the most infected sites are the dorsal head region and the caudal peduncle.

The analysis of incidence illustrates the infection patterns in male and female fish (Fig. 2). Significantly ($p < 0.05$) more infected areas in males are similar to those predicted on the basis of the composite image. The dorsal body surface stretching from the operculum with the proximal end of the dorsal fin to its posterior end, the adipose fin, a small medial part of the caudal fin, the posterior end of the anal fin and its base, and the bases of pelvic fins are more often infected than the rest of the body. In females, the corresponding areas are: the dorsal head region stretching from the upper jaw to the operculum, the dorsal body surface located anteriorly to the proximal end of the dorsal fin, the caudal peduncle and the fin, the posterior end of the anal fin and its surroundings stretching dorsally to the lateral line, the proximal posterior end of the pelvic fins and the leading edge of the pectoral fins.

The analysis of incidence also allows a comparison of uninfected areas between sexes (Fig. 2). In males, the region of the lower jaw, the ventral part of the operculum, and the dorsal and ventral edges of the caudal fin are most frequently free of water mold growth. In females, the ventral part of the operculum, flanks, and the tips of the dorsal, pelvic, anal and caudal fins are most commonly uninfected.

Statistical comparison of infection patterns between sexes shows that the dorsal part of the operculum, the dorsal and ventral parts of the flanks, and the dorsal, adipose, anal, pelvic and pectoral fins of the male are significantly more often infected than in females (Fig. 3). The dorsal half of the caudal fin and the area adjacent to the nostrils are more commonly infected in females than in males.

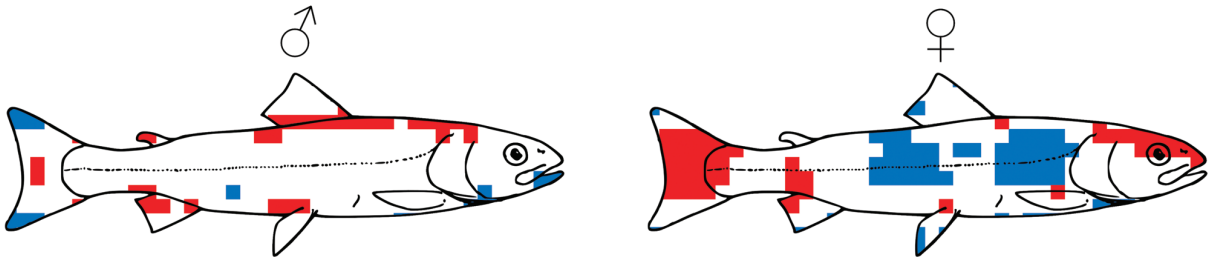

Figure 2

Patterns of *Saprolegnia* spp. infected and uninfected areas of the fish body, plotted for males and females of UDN-affected sea trout. Areas marked red are significantly more frequently infected ($\chi^2 > 3.841$; $p < 0.05$). Areas marked blue are significantly less frequently infected ($\chi^2 > 3.841$; $p < 0.05$).

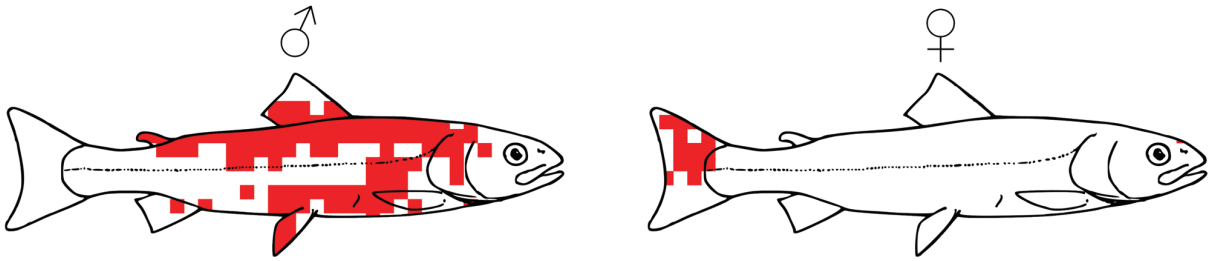

Figure 3

Comparison of infection patterns between males and females of UDN-affected sea trout with the 2 × 2 Contingency Chi-square test. Marked areas are significantly ($p < 0.05$) more often infected secondarily with *Saprolegnia* spp.

Discussion

In this work, the research material was not subjected to microbiological evaluation. Based on the results presented by previous researchers and on gross pathology, we assumed that secondary infections in UDN-affected sea trout (*Salmo trutta* m. *trutta*) from the Słupia River were caused by water molds belonging to the *Saprolegnia* genus (Kazuń et al. 2011).

Several predisposing factors are linked to the oomycete infection development. Some of them act on fish and some on the pathogen itself. The combination of these factors prompts infection (Roberts 2012). Saprolegniasis affects salmonids during freshwater stages of their life cycle. *Saprolegnia* cannot survive in seawater, therefore marine stages of anadromous salmonid fish are free of infection (Bruno et al. 2011).

There are literature reports on the seasonality of *Saprolegnia* infections. In most cases, infection occurs in autumn and winter, when water temperature is low and/or drops by 6–10°C within 24 hours (Bly et al. 1993). In the Słupia River, fish with signs of infection have been observed from 2007 between late October and mid-December (Bartel et al. 2009; Grudniewska et al. 2011; 2012; Kazuń et al. 2011).

The correlation between sexual maturation of salmonids and *Saprolegnia* infection has been documented (Fregeneda-Grandes et al. 2008; Roberts 2012). The process of sexual maturation in brown trout is followed by a decrease in mucous cell concentration and reduction in the number of epithelial cell layers during spawning. These changes particularly affect males and were observed in anadromous and non-migratory fish forms. The mucous layer and epidermis establish the first, immediate, barrier against potential pathogens occurring in the environment (Pickering 1977). Admission of androgen promotes the occurrence of infection in salmonids. Cross & Willoughby 1989 in their experiment implanted the rainbow trout, *Oncorhynchus mykiss* (Walbaum), with the androgen 11-ketotestosterone. During the experimental challenge with *Saprolegnia*, they observed increased retention of zoospores on the external surface of fish treated previously with the sex hormone. Furthermore, *Saprolegnia* susceptibility seems to be associated with immunosuppression observed in mature salmonids during spawning. In chinook salmon, *Oncorhynchus tshawytscha*

(Walbaum), physiological testosterone concentrations are known to kill leucocytes in vitro. It acts directly on leukocytes via the androgen receptor on their surface. This action leads to the death of a significant number of white blood cells (Slater & Schreck 1997). Lymphocytopenia in brown trout was observed in both sexes during the entire spawning season. The greatest reduction in the number of lymphocytes occurs between October and November (Pickering 1986). In 2007 and 2008, more male sea trout with visible infection symptoms were observed in the survey of rivers in northern Poland than females (Bartel et al. 2009). The role of cortisol in *Saprolegnia* infection susceptibility is well described. In salmonids, the increased levels of blood plasma corticosteroids, caused by stress, can result in immunosuppression (Pickering 1984). There is also evidence that during sexual maturation, steroid metabolic changes occur directly in the epidermis (Pickering & Pottinger 1985; 1987). *Saprolegnia* triggers a severe inflammatory response in fish (induction of pro-inflammatory cytokines and antimicrobial peptides), caused by its cell wall components, and severely suppresses gene expression associated with fish adaptive immunity (Belmonte et al. 2014).

Areas of damaged skin are most commonly targeted by *Saprolegnia* infections (Bruno et al. 2011; Roberts 2012; Van Den Berg et al. 2013). Even slight loss in epidermis continuity promotes oomycete invasion. There is a proven correlation between saprolegniasis and ectoparasite infestation (e.g. Ichthyophthirius, Costia, Gyrodactylus, Scyphidia). In mature brown trout, particularly males, the ectoparasite infestation is more frequent and severe than in immature fish (Pickering & Willoughby 1982; Richards & Pickering 1978). The *Saprolegnia* mycelium can produce motile zoospores that, after encystation, can attach to a host and germinate, thus spreading the infection in populations (Van West 2006). The UDN itself is also thought to be a factor predisposing fish to *Saprolegnia* infection. During the first stages of UDN, oomycete infection is absent. The first visible small, greyish ulcers are found on the head and in the operculum region. Progressive Malpighian cell degeneration leads to complete epidermis destruction. Eventually, only the epidermal basement membrane persists and oomycete infection is inevitable (Roberts 1993).

The infection pattern of males obtained in the present study is different from that of female fish (Fig. 1). Males are more prone to infection on the dorsal region, including the proximal part of the dorsal fin and adipose fin. Infection of these areas can be explained by the sexual dimorphism in cellular skin composition between sexes observed during the spawning season. Reduction in the number of goblet cells in spawning male fish was reported by Pickering (1977). Since mucus, produced by goblet cells, is the first protective barrier against many infectious agents occurring in freshwater environments, we suggest that a decrease in its production can increase infection susceptibility in mature brown trout. In female fish, the mycelial growth was observed most commonly on the peduncle and caudal fin. During the spawning period, female brown trout make abortive attempts at building a redd. Fish lay on the side of the watercourse bottom and with rapid tail movements try to dig a redd. These are thought to be responsible for epithelial damage promoting peduncle and tail fin *Saprolegnia* infection (Richards & Pickering 1978). Similar patterns were observed in wild brown trout from León province rivers (Spain) (Fregeneda-Grandes et al. 2001) and Loch Leven (Scotland) (Richards & Pickering 1978). Infection in the dorsal head region stretching from the upper jaw to the operculum in female brown trout was not reported previously. The UDN development is characterized by epidermis cell destruction, especially in the head and operculum region (Roberts 1993). Skin damage is one of the predisposing factors for oomycete infections and is followed by rapid development of infection symptoms (Bruno et al. 2011; Roberts 2012; Van Den Berg et al. 2013). However, the severity of infection in the head region in this case cannot be explained by UDN alone. During artificial spawning, female fish were kept briefly in large, flow-through concrete pools supplied with river water. Fish gathered near the pool water outlet secured with steel bars, therefore abrasion from contact with these bars and other specimens could have occurred, leading to pathogen invasion.

Significantly less frequently infected areas in males are the lower jaw and the ventral part of the operculum. This is consistent with Richards & Pickering (1978) observations. Uninfected areas on the dorsal and ventral edges of the caudal fin in males are somewhat unexpected and have not been previously mentioned in the literature. There are two large areas on female fish flanks where the infection is absent. The ventral part of the operculum is also usually uninfected, as in the case of males. Interestingly, this pattern is similar to that of hatchery-reared fish described previously by Richards & Pickering (1978), and not that of wild fish.

There are visible differences in infection patterns between male and female fish (Fig. 3). Large areas of flanks are significantly more frequently infected in males. In females, the dorsal half of the tail fin is most commonly affected by oomycete mycelia. Our observations are in agreement with those of Richards

& Pickering (1978). A more uniform distribution of secondary infection on the male fish body can be caused by abrasions received during territorial defense and spawning behavior. In the case of female brown trout, infection of the tail fin can be linked to abortive attempts at building redds.

The presented results show that there are different patterns of secondary infection for the UDN-affected male and female sea trout from the Słupia River, which are associated with sexual dimorphism in the skin structure of sexually mature sea trout. The obtained results are related to many infection predisposing factors that have an impact on the oomycete infection. Visualized infection patterns can be helpful in macroscopic diagnosis of early secondary infection stages by identifying the spots with the highest probability of infection on the fish body, not only in the sea trout but also in other salmonids. We believe that it is necessary to determine etiological factors and treatment for the cause of secondary infections – UDN. This knowledge is essential for developing effective prevention strategies against a disease threatening wild and cultured salmonid populations.

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