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SHORT PAPER

# A Preliminary Study on Protease Activity of Russian Sturgeon, *Acipencer gueldenstaedtii* Brandt and Ratzenburg, 1833, at Early Life Stages

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**Abstract**

The fish feed industry continues to researches for optimum diet demands for candidate species culture. For this purpose, *in vitro* analyze methods may be more efficient than *in vivo* assays. This study includes two different stages; the first one is about the effect of commercial feeding protocol on protease enzyme alteration and the second one is about the inhibitory effects of different protein sources on early life proteases of Russian sturgeon, *A. gueldenstaedtii*, juveniles. In the first step, feeding with live prey and transition period to artificial feed significantly affected the daily amount of protease in digestive system ($P<0.05$). In the second part, some protein sources used in micro diets were tested *in vitro* for examination of their possible inhibitory effects on the proteases of Russian sturgeon larvae. The minimum inhibitory effect was obtained from fish meal (15.44%), but however, soybean protein concentration, soybean meal, corn gluten and rice bran inhibited the proteases significantly higher than FM and its combinations (63.55, 71.81, 72.24, and 80.77%, respectively). Additionally, dual combinations between fish meal and soybean meal/soybean protein concentration with the ration of three to one (3:1) was moderate (26.38 and 22.13 %), whereas blood meal extremely produced a 97.28% inhibitory ratio.

*Keywords*: Russian sturgeon, protease activity, inhibition, feed ingredients.

**Rus Mersin Balığının, *Acipencer gueldenstaedtii* Brandt ve Ratzenburg, 1833, Yaşamlarının İlk Dönemlerindeki Proteaz Aktivitesi Üzerine Bir Ön Çalışma**

**Özet**

Balık yemi endüstrisi, aday türlerin optimum rasyon ihtiyacını belirlemek için araştırmalarına devam etmektedir. Bu amaçla yapılan *in vitro* analiz metotları, *in vivo* metotlara göre daha verimli olabilir. Bu çalışma iki farklı çalışmadan oluşmaktadır; bunlardan birincisi ticari besleme protokolünün proteaz enzimlerinin değişimi üzerine olan etkisini, ikinci çalışma ise farklı protein kaynaklarının Rus mersini, *A. gueldenstaedtii*, jüvenillerinin erken dönem proteaz aktivitiesi üzerine olan inhibisyon etkisini belirlemeyi amaçlamıştır. İlk çalışmada, canlı yem ile besleme ve karma yeme geçiş periyodunun, sindirim sistemindeki günlük proteaz miktarını istatistiki olarak etkilediği tespit edilmiştir ($P<0.05$). İkinci çalışmada ise, mikro yemlerde kullanılan bazı protein kaynakları, Rus mersini jüvenillerinin proteaz enzimlerini olası inhibe edici etkilerini belirlemek için *in vitro* olarak test edilmiştir. En düşük inhibisyon etkisi balık ununda (%15.44) gözlemlenmiş, ancak buna karşın soya protein konsatresi, soya unu, mısır glüten ve pirinç kepeği proteaz enzimlerini balık unu ve diğer hammaddelerle yaptığı kombinasyonlardan istatistiki açıdan daha yüksek oranda inhibe etmiştir (sırasıyla % 63.55, 71.81, 72.24 ve 80.77). Buna ek olarak, balık ununun soya unu/soya protein konsantresi ile 1:3 oranındaki kombinasyonları kabul edilebilir seviyede inhibe edici bulunurken (%26.38 ve 22.13), kan ununun aşırı seviyede inhibe edici olduğu tespit edilmiştir (%97.28).

*Anahtar Kelimeler*: Rus mersini, proteaz aktivitesi, inhibisyon, yem hammaddeleri.

## Introduction

Sturgeons are one of the oldest living vertebrates and generally called "living fossils". Populations are found primarily in cold and temperate regions of the Northern hemisphere. All sturgeon species are endangered due to overfishing, river damming, regulation, deepening and strengthening of river banks (Napora-Rutkowski *et al*., 2009). Interest in sturgeon species mainly focused on caviar, smoked fish, soup, isinglass (collagen), ivory and leather.

Although endogenous production of digestive enzymes by larvae is emphasized to be adequate for exogenous live or artificial feeds (Hofer and Köck, 1989; Kolkovski, 2001), the selection of the most suitable feed ingredients for microfeeds and

manufacturing are very important for candidate specie's culture. Recently, the plant protein sources were intensively started to be used in commercial fish feeds instead of fishmeal to protect natural fish populations which are exploited for fishmeal production. On the other side, fish farmers complain for high feed conversion ratios and low growth performance. It is thought that this situation is probably caused by plant protein sources due to having inadequate amino acid profiles (Halver and Hardy, 2002).

Aquaculture research results concerning breeding, larval rearing and digestive enzymes were already presented for some Acipencer species (Willot *et al*., 2001; Gawlicka *et al*., 2002; Furne *et al*., 2005; Napora-Rutkowski *et al*., 2009; Babaei *et al*., 2011; Sanz *et al*., 2011; Noori *et al*., 2012) and commercial feeds are already produced by some companies. However, the effect of commercial feeding protocol on enzyme activity, inhibitory effects and bioavailability of combination of different protein sources on protease activity of Russian sturgeon is still obscure. In the present work, in vitro methods were examined to assess the variance of protease levels according to feed types and the interaction between proteases and different protein sources that can be used in preparation of microfeeds for Russian sturgeon.

## Materials and Methods

### Source of Fry

Fertilized sturgeon eggs were imported from Russia and hatched in commercial fish farm in Homokmègy, Hungary (Rideg & Rideg Ltd.). After yolk sac absorption, larvae were transferred and stocked into recirculation system. The water temperature was 17±1°C during experiment. Commercial feeding protocol (Figure 1) was applied for 30 days and 30 days-old larvae were sampled into 3 ml eppendorf tubes. Samples were rinsed in distilled water and stored at −80°C until protease and inhibition analyses. The whole body of larvae were homogenized to get enzyme extracts by centrifugation (12000 g, 20 min, 4°C).

### Determination of Protease Activities

Casein (10 mg $ml^{-1}$) was used as substrate and dissolved in 50 mM Tris-HCl buffer at pH 9 (Walter, 1984). The substrate and the enzyme extracts of larvae were incubated at 37°C for 1 hour. Then, 500 µl trichloroacetic acid (TCA) (120 g $L^{-1}$) was used to stop the reaction. The mixture was incubated at 4°C for 10 minutes and after centrifugation (8000 g, 15 min, 4°C) the absorbance was recorded at 280 nm. One unit of enzyme activity was defined as 1 µg of tyrosine released per minute (Hazman and Gökçek, 2014). The soluble protein concentrations of larvae were determined according to Bradford (1976).

### Determination of Inhibition Effects of Protein Sources on Proteolitic Activity

A modified method of Garcia-Carreno (1996) was used to determine inhibition effects of fish meal (FM), soybean meal (SM), soybean protein concentration (SPC), corn gluten (CG), rice bran (RB), blood meal (BM) and combination of FM/SM (1:3, 1:1, 3:1), FM/SPC (1:3, 1:1, 3:1) and SM/MG (1:3, 1:1, 3:1) as protein sources on protease activity of 30 day-old juvenile. Different feed ingredients and protease enzyme extracts were pre-incubated and the residual activity was measured. The extracts (20 µL) were incubated with protein solutions (20 µL) in HCl Tris buffer (500 µL) for 60 min at 25°C (pH 9.0). In the control group, same amount of distilled water was used instead of protein solutions in the mixture. Afterwards, 100 µL casein was added and the mixture was incubated for 120 min at room temperature. Finally, 500 µL TCA (120 gL-1) was added to stop reaction and after centrifugation (8000 g, 15 min, 4°C) the absorbance was recorded at 280 nm. TCA was added prior to casein in blanks (Alarcon *et al*., 1999).

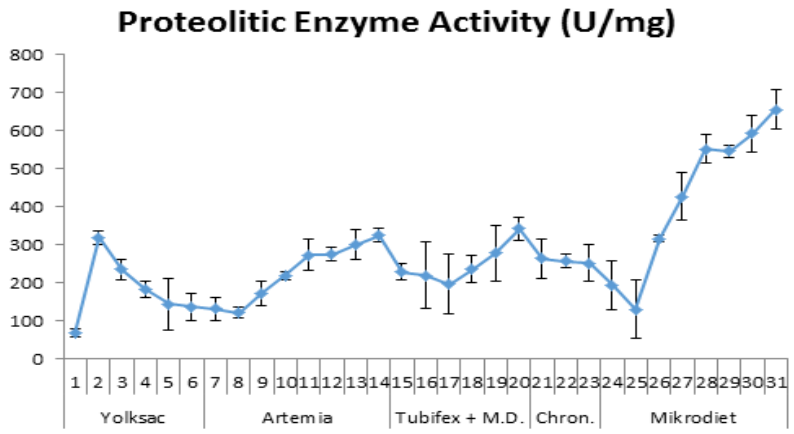


**Figure 1.** Feeding protocol and daily chances of digestive protease activity on Russian sturgeon at first month.

**Statistical Methods**

Kolmogorov-Smirnov normality test and Bartlett's test for homogeneity were used prior to comparison of data. A one-way ANOVA test was applied for comparisons by using SPSS 15.0 (Hazman and Gökçek, 2014).

## Results

The feeding protocol and daily chances of digestive protease activity on Russian sturgeon pre-post larvae and juveniles were presented in Figure 1. By the possible increasing of cytosolic enzyme secretion on 2nd day after hatching (DAH), the protease activity reached to the highest level in the yolk sac stage. Then, the enzyme activity decreased to the minimum level by decreasing of the total mass of yolk sac. Exogenous feeding with Artemia directly affected the enzyme activity and it increased till the DAH 14. After this point, post larvae were fed with tubifex+M.D. mixture, however larvae unwillingly accepted this new feed type between DAH 15th and 18th. Although larvae were corresponded to new feed habit, the same situation was observed on the feeding period with Chironomid+M.D. between DAH 21st and 23rd. The second lowest enzyme secretion was obtained on the DAH 25. By the development of digestive tract, microdiet feed was accepted by juveniles and the total protease activity started to increase after DAH 26th.

The protease activity was measured 592.74±47.61 U/mg on 30 days-old fry. The inhibitory degree of feed ingredients on protease activity was given in Table 1. The lowest inhibition was observed from FM (15.44±5.59%) and inhibition produced by other protein sources were relatively high. Combination of two different feed ingredients inhibited relatively higher such as 26.38±2.02 and 22.13±2.69% from FM/SM (3:1) and FM/SPC (3:1), respectively. The highest inhibition of juvenile protease activity was induced by BM (97.28±2.31).

## Discussion

In this study, the mouth of Russian sturgeon larvae was opened on 9th day and the protease activity was increased by the presence of Artemia in the culture medium. Sanz *et al*. (2011) stated that although the acidic protease activity were detected at the beginning of the exogenous feeding, the increase of enzyme activity should be exogenous originated because gastric glands didn't have the granules of zymogen secretion in A. naccarii yet. Controversially, it has been suggested by several researchers that exogenous enzymes are unnecessary for digestion in the early larval stage (Segner *et al*., 1993; Kim *et al*., 2001) but the live food may trigger the secretion of proteases (Zambanino Infante and Cahu, 2001). Afterwards, feeding firstly with tubifex+ M.D. and Chironomids+M.D. negatively affected the digestion and absorption capabilities due to possibly inadequate enzyme secretion (Faulk *et al*., 2007). Then, the protease activity was on the rise in an orderly manner after DAH 25. This is because the juvenile developed anatomically similar digestive system as like adults (Sanz *et al*., 2011).

The present study demonstrated that BM significantly reduced (97.28%) the activity of digestive proteases in 30 day-old Russian sturgeon. On the other, FM showed the lowest (15.44%) inhibitory effect on protease activity. Alptekin (2016) stated the same results for FM (15.34%) in Siberian sturgeon, A. baerii. In their study, there were no statistical differences between FM and SPC ($P<0.05$), even though the SPC showed a lower inhibitory effect on protease activity (14.45%). In the present study, all other single ingredients negatively affected the digestive activity in contrast to FM. Similar results were obtained when commercial ingredients were

**Table 1.** Protein sources and inhibition degree of protease activity

| Protein Sources | Inhibition degree of protease activity (± SD) |
|---|---|
| Fish Meal (FM)(69% CP) | $15.44\pm5.59^{a}$ |
| Soybean Meal (SM)(48% CP) | $71.81\pm3.82^{e}$ |
| Soybean Protein Concentration (SPC) (58% CP) | $63.55\pm5.46^{de}$ |
| Corn Gluten (CG)(60% CP) | $72.24\pm4.17^{e}$ |
| Rice Bran (RB)(n/a) | $80.77\pm2.28^{f}$ |
| Blood Meal (BM)(75.5% CP) | $97.28\pm2.31^{g}$ |
| FM/SM (1:3) | $76.81\pm4.39^{ef}$ |
| FM/SM (1:1) | $59.24\pm8.45^{d}$ |
| FM/SM (3:1) | $26.38\pm2.02^{b}$ |
| FM/SPC (1:3) | $62.46\pm6.35^{d}$ |
| FM/SPC (1:1) | $35.75\pm5.67^{c}$ |
| FM/SPC (3:1) | $22.13\pm2.69^{b}$ |
| SM/CG (1:3) | $69.02\pm6.92^{d}$ |
| SM/CG (1:1) | $70.85\pm9.89^{de}$ |
| SM/CG (3:1) | $76.95\pm4.52^{ef}$ |

n/a: not available

Means with the same superscripts are not significantly different ($P<0.05$)

tested for northern pike juveniles (Töre *et al*., 2014). Whereas, the combination of FM/SM (3:1) and FM/SPC (3:1) showed acceptable results (26.38 and 22.13 %, respectively). By increasing of FM ration in both mixture, the inhibition effect clearly reduced probably due to the changing of amino acid composition.

Micro feeds for Sturgeons are already commercially produced by several feed companies. Although these feeds are accepted by fish, sometimes poor growing or nutritional originated health problems can be occurred. This is probably due to undeveloped digestive tract in early stages of larvae or juveniles. On the other hand, some researchers claimed that some specie' digestive enzyme secretion, especially a high protease and amylase activity, were already existed since early stage of development (Cahu and Zambonino, 1995; Moyano *et al*., 1996) and it has been hypothesized that this is due to inhibition of enzymes by inadequate protein sources (Alarcon *et al*., 1999). Also, limited intake of amino acids in charged to produce new body proteins may be caused the presence of enzyme inhibitors in artificial feeds (Garcia-Carreno, 1996). In this study, no reduction were obtained, even though dry air were used to extinguish possible inhibitory. By this kind of in vitro assays, the protein sources in used for adult fish (Grabner 1985; Dimes *et al*., 1994) and shrimp (Lana and Pan, 1993; Ezquerra, 1997) were already evaluated to prove that these ingredients are useful or not, however this is the first evaluation for a sturgeon specie.

In conclusion, according to analyze results, feeding larvae with Tubifex and Chronomids without M.D. supplementation before DAH 25 can give better results for adequate secretion of proteases. Also, it is clear that the plant protein sources were negatively affected protease activity on DAH 30. The information from in vitro assays may preliminary provide an explanation for why plant protein sources gave poor results for Russian sturgeons. However, to get a better knowledge about the effect of different feed ingredients, the determination of the degree of hydrolysis and sequential analysis of protein hydrolysis products should be examined in the future.

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