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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

SHORT COMMUNICATION KISA MAKALE

PRESENCE OF GENUS *PTEROIS* (Oken, 1817) (Scorpaeniformes, Scorpaenidae): EXTENSION OF INVASIVE RANGE IN CARIBBEAN SEA AND FIRST PUBLISHED RECORD FOR LOS FRAILES ARCHIPELAGO

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Abstract: The present work reports the presence of Lionfish (*Pterois volitans*) in Los Frailes Archipelago, Venezuela. This is the first published record of Lionfish presence for this location and the extension of their invasive range to the South-East of the Caribbean Sea.

Keywords: Lionfish, Invasive range expansion, Caribbean Sea, Los Frailes Archipelago

Öz: ***Pterois* (Oken, 1817) (Scorpaeniformes, Scorpaenidae) Genusunun Bulunması Üzerine: Karaib Denizinde İstila Alanının Genişlemesi ve Los Frailes Takımadaları İçin Yayınlanan İlk Kayıt**

Bu çalışma, bir İskorpit balığı türü olan *Pterois volitans*, Venezuela Los Frailes takımadalarındaki varlığını bildirmektedir. Karayip Denizi'nin güneybatı sınırındaki bu bölge için *Pterois volitans*'ın varlığı ve istilacı yayılım alanını genişletmesi açısından yayınlanmış ilk kayıttır.

Anahtar Kelimeler: İskorpit, İstila alanı genişlemesi, Karaib Denizi, Los Frailes Takımadaları

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Introduction

Lionfishes (family Scorpaenidae) are the first marine fishes to invade the Western Atlantic Ocean and Caribbean Sea; both the Red Lionfish *Pterois volitans* (Linnaeus, 1758) and the Devil Firefish *Pterois miles* (Bennett, 1828) have established in this region, beyond their native range in the Indo-Pacific, and spread with remarkable speed (Hamner et al. 2007; Schofield 2010). *Pterois miles* is usually less abundant and studies based on analysis of mitochondrial cyt b, have demonstrated that only 7% of specimens analyzed in the invaded area represented that species, identifying the other 93% as *P. volitans* (Hamner et al. 2007).

The first documented capture of lionfish in the Atlantic waters was in 1985 southeastern of the Florida Peninsula (Morris and Akins 2009). Later in 1992, after Hurricane Andrew, a private aquarium was destroyed releasing several lionfish into Biscayne Bay, Florida (Hare and Whitfield 2003). Since then, several authors have confirmed an alarming dispersion along the Gulf of Mexico (Aguilar-Perera and Tuz-Sulub 2010; Santander-Monsalvo et al. 2012), the northwestern Atlantic Ocean and the Caribean Sea (Guerrero and Franco 2008; Lasso-Alcalá and Posada 2010; Alexander and Haynes 2011). In 2010, Schofield dated the geographic spread of lionfishes using USGS-NAS database not differentiating between the two invasive species of *Pterois* genera (*P. volitans* and *P. miles*).

Materials and Methods

In this context, a specimen of Pterois volitans was photographed and recorded *in situ* underwater during SCUBA dives in Los Frailes Archipelago in July 2011 (Figure 1). The species was identified retrospectively using the original description, video and photographs, alongside with other published photographs (Schultz 1986; Froese and Pauly 2012). Due to the lack of a permit this specimen was not collected.

Results and Discussion

This is the first published record with photograph of Lionfish (*Pterois volitans*) presence for this location and the extension of their invasive range to the south-east of the Caribbean Sea (Figure 2).

Acknowledgments

Authors are indebted to the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and to Martin Rada and people from the Science Museum of Caracas and the Marine Museum of Margarita, Venezuela for assistance on species identification.

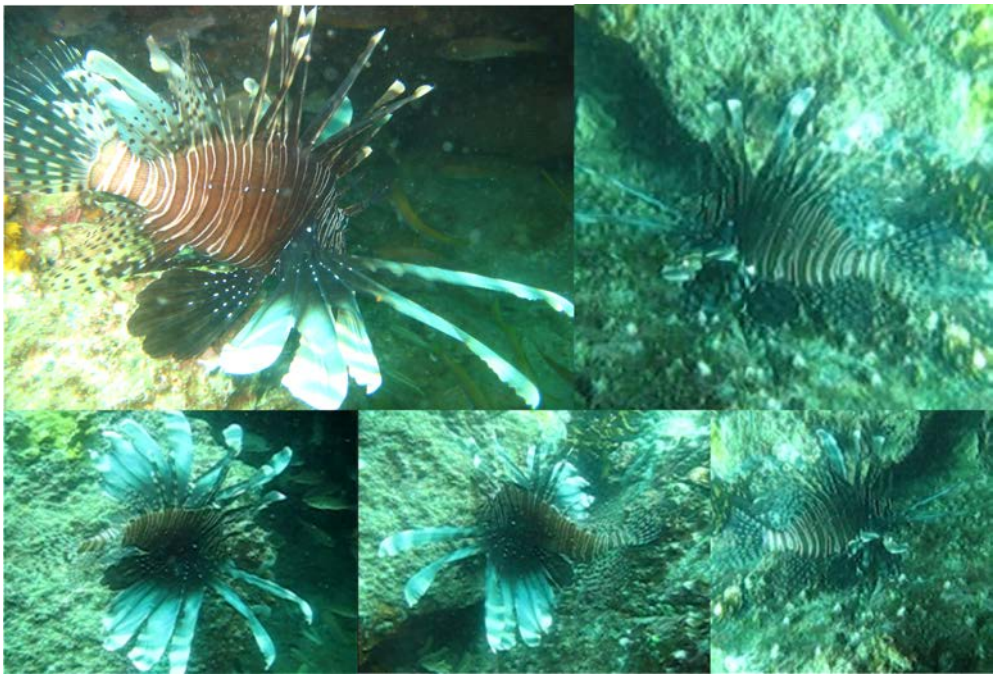

Figure 1. Photographs taken of a specimen of Lionfish (*Pterois volitans*) during SCUBA dives in Los Frailes Archipelago, Venezuela.

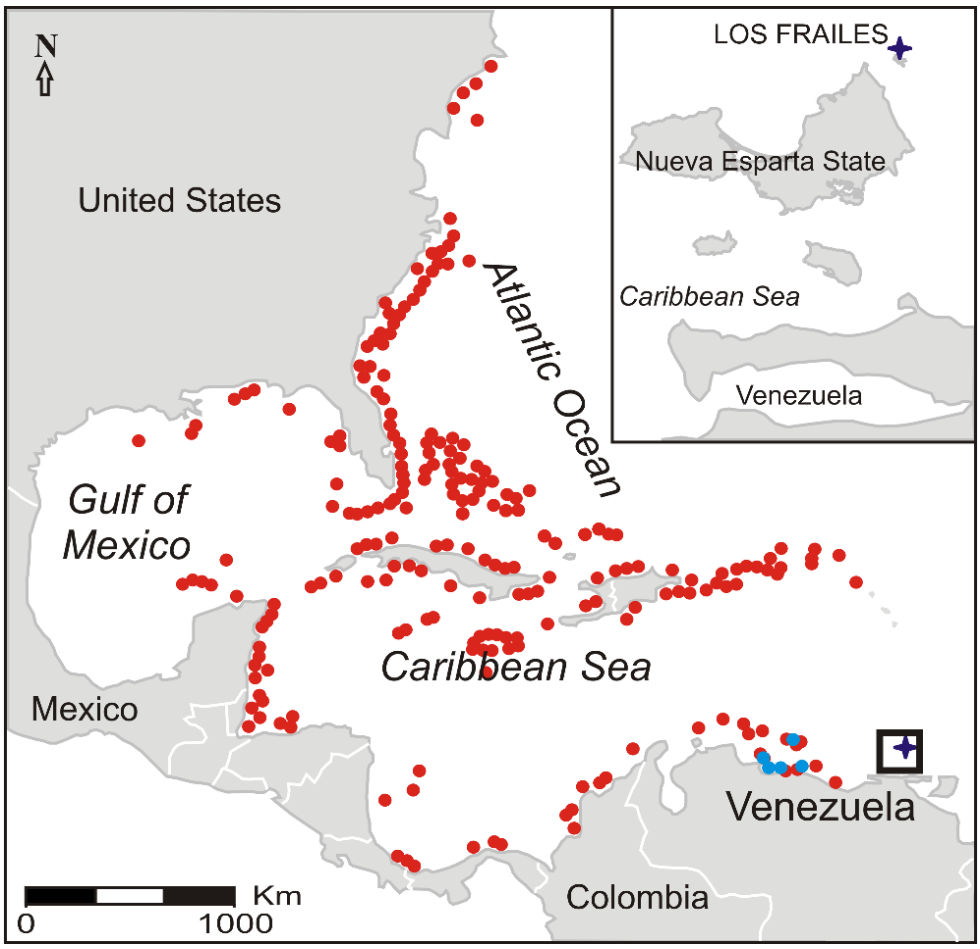

Figure 2. Range expansion of Lionfish (*Pterois* sp.) in the Caribbean Sea and Western Atlantic Ocean. Red circles show confirmed Lionfish (*Pterois* sp.) occurrences in the northwestern Atlantic and Caribbean Sea (Schofield 2010); Light blue circles show confirmed *Pterois volitans* on the coast of Venezuela (Lasso-Alcalá and Posada 2010); and the blue star represents the new South-Eastern record for the Caribbean Sea.

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

SHORT COMMUNICATION KISA MAKALE

THE FIRST RECORD FOR WATERMITE *Arrenurus berolinensis* FROM TURKEY, (ACARI: HYDRACHNIDIA)

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Abstract: In this study, a very rare water mite *Arrenurus berolinenis* is described for the first time from Turkey. The species was previously reported from Eastern Prussia (1907), Berlin (1896) and Overijssel (2007). The first description of the female was reported by Smit (2007). We have been reporting *Arrenurus berolinenis* from Turkey as the fourth report. Here, we have discussed the characteristics of this species by giving measured values of male specimens.

Keywords: *Arrenurus berolinensis*, Water mite, New record, Turkey

Öz: **Su Kenesi *Arrenurus Berolinensis* İçin Türkiye'den İlk Kayıt, (Acari: Hydrachnidia)**

Bu çalışmada, çok nadir bir su kenesi olan *Arrenurus beroliensis*, Türkiye'den ilk defa tanımlanmıştır. Tür daha önce Doğu Prusya (1907), Berlin (1986) ve Overjussel (2007)'den rapor edilmiştir. İlk dişi tanımlaması Smit (2007) tarafından rapor edilmiştir. *Arrenurus berolienses*'in Türkiye'den dördüncü kaydını rapor etmekteyiz. Burada erkek tür için, ölçülen ve gözlenen karakterlerinin tartışmaları yapılmıştır.

Anahtar Kelimeler: *Arrenurus beroliensis*, Su kenesi, Yeni kayıt, Türkiye

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Introduction

Arrenurus berolinensis Protz, 1896 is a very rare water mite, previously known from three localities only. Protz (1896) described the species first time from Fenn of the Grunewaldsee near Berlin. Therefore, the species' name is reminding of Berlin (= Berolinum). Second, Viets (1911) reported it from Perwilten in Eastern Prussia, nowadays in the Russian enclave Kaliningrad, where the species had been collected in September 1907. However, both records were only reported for male specimens, and no finding of a female specimen has been reported. As third record, four males and one female were collected from the northwest of the province of Overijssel, the Netherlands and the first description of the female was done by Smit (2007). In the present study, we have collected four male specimens of *A. berolinensis* from Karakuyu Lake in Dinar District of Afyonkarahisar Province of Turkey.

Materials and Methods

In the present study, four male individuals were examined. The samples obtained from the lake of Karakuyu, province of Afyonkarahisar, Turkey, N 38° 03' E 30° 14'. All the samples were deposited in the Zoological Laboratory of the University of Afyon Kocatepe (Figure 1).

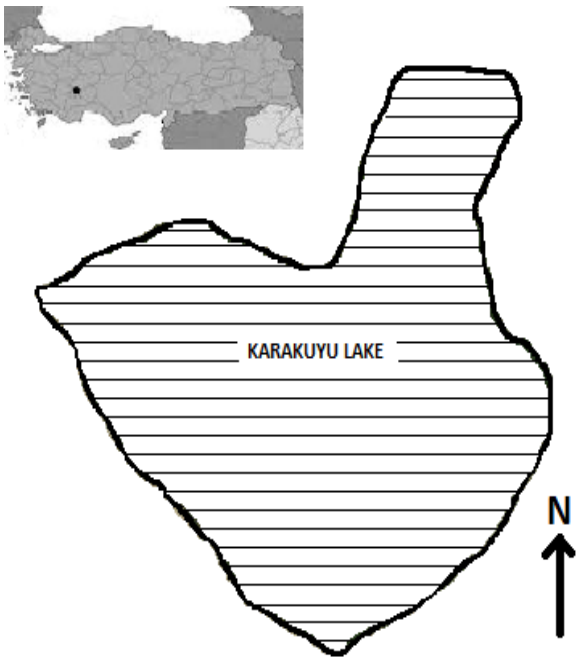

Figure 1. Karakuyu lake

Results and Discussion

Arrenurus berolinensis

Description

Male: Idiosoma 981 µm long (without petiole) and 899 µm wide, including petiole 1321 µm long (Figure 2). Body color greenish-brownish. Anterior idiosoma margin concave. Dorsal furrow completed extending onto lateral sides of ideosoma. Petiole very long, with two curves satea anteriorly. Petiole ending in some hooked extensions, like many swimming setae. The distance from the front edge of dorsal furrow 190 µm. Capitulum 155 µm, chelicerae 167 µm and nail 72 µm in length. The distribution parts of the setae in the form of palp; 1, 2 and 3. There are long setae on the palps. The upper parts of palp; 32-55-60-57-32 = 236 µm in length, the lower parts; 22-32-22-52-27=155 µm in length and the palp heights; 30-55-57-52-10 µm in length. Coxae lengths; 270-250-310, 380 µm. Genital plates narrowed towards the sides of the body width of the genital plates 650 µm. Genital opening is 70 um. There are swimming hairs on the legs and there is a protrusion on the leg of fourth. The lengths of legs; LegI: 70-90-110-160-180-230 = 840 µm, LegII: 80-90-100-180-210-250-140-160= 960 µm, LegIII: 90-120-170-150-120-180= 830 µm and legIV: 120-150-180-250-140-160 = 1000 µm.

For the male specimens of *Arrenurus berolinensis*, the length and width of Idiosoma were reported as 960 µm (without petiole) and 899 µm, respectively (Smith 2007). In addition, Idiosoma plus petiole, that is the body length, was 1252 µm as the length of petiole was 389 µm (Smith 2007). These measures are almost similar what we have determined for *A. berolinensis* male specimens collected from the Karakuyu Lake. In addition, Smit (2007) reported that dorsal furrow is incomplete and not extending to the lateral sides in male specimens. On the other hand, the dorsal furrow is clearly complete, that is it extends to the lateral sites. Although genital plate completely surrounds the sexual plate in our male samples, genital plate of Smith's (2007) male specimens does not surround the sexual opening. The length of Petiole and its appearance is similar to a large extend by the Smit's specimen. In terms of other properties, there is not much differences between our samples and Smit's samples.

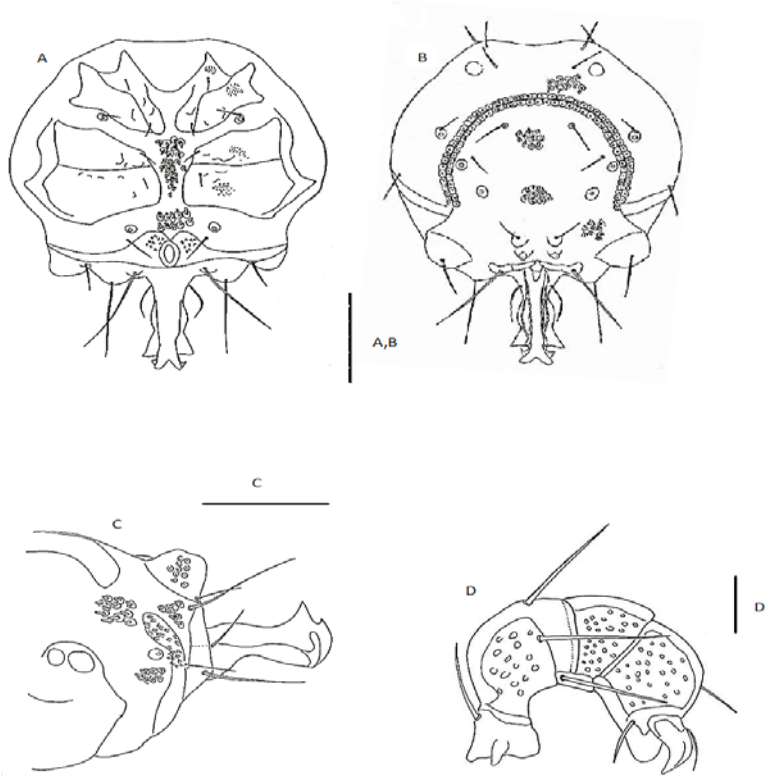

Figure 2. *Arrenurus berolinensis*, A. ventral view male, B. dorsal view male, C. lateral view male. D) Palp Scale bar: A,B) 250 µm C) 340 µm D) 50 µm

The length and width of Idiosoma for female specimen of *A. berolinensis* were reported as much as 1166 µm and 1085 µm, respectively. In addition, the front edges of Idiosoma were shown as a slight concave for both male and female specimens (Smit 2007). This is also the case for our specimens.

Males of this extraordinary species are relatively easy to identify with outstanding features of their Palps. On the other hand, this diagnose is very difficult for females (Smit 2007).

Conclusions

Although there are very few records about this species, it is seen that *A. berolinensis* mostly lives in the muddy swamp areas. Almost all of the collected specimens were living in peat pits having neutral pH and low nutrient and mineral content. Uncommonness of such kind of areas could be explanation of why this species reported rarely. Morphology, body measurements and habitat characteristics of our specimens are largely similar to previous records.

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

KARYOMORPHOLOGY OF THREE INDIAN MAJOR CARPS FROM HARYANA, INDIA

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Abstract: The purpose of the present study was to investigate and compare the karyotypes of three Indian major carps, *Catla catla* (Hamilton, 1822), *Labeo rohita* (Hamilton, 1822), *Cirrhinus mrigla* (Hamilton, 1822) in terms of chromosomal architechture, karyotype formula and number of chromosomes from aquatic ecosystems of Haryana, India. Karyotypes of these carps were investigated by examining metaphase chromosomes. The results indicated that the diploid (2n) chromosome number of all the three major carps was 50. *Catla catla* consisted of 22 acrocentric, 2 subtelocentric, 20 submetacentric and 6 metacentric chromosomes. *Labeo rohita* consisted of 32 acrocentric, 4 subtelocentric, 6 submetacentric, 8 metacentric while *Cirrhinus mrigla* consisted of 30 acrocentric, 8 subtelocentric, 6 submetacentric, 6 metacentric chromosomes. Centromeric Index, arm ratio and fundamental number was also determined. No heteromorphic sex chromosomes were cytologically detected. The variability in size, shape and arm number (NF) of chromosomes among these three species suggest that diversification in these fish species of same family is related to structural changes in chromosomes. Variations in karyotype formulae are also observed with respect to earlier studies which may be due to variations in habitat conditions as a result of anthropogenic activities.

Keywords: *Catla catla*, Chromosome, *Cirrhinus mrigla*, Karyotype, *Labeo rohita*

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Öz: **Hindistan, Haryana bölgesinden üç ana sazan türünün karyomorfolojisi**

Çalışmanın amacı, Hindistanın Haryana su ekosistemlerinde bulunan başlıca üç sazan türü olan, Catla catla (Hamilton, 1822), Labeo rohita (Hamilton 1822) ve Cirrhinus mrigla (Hamilton, 1822) türlerinin karyotiplerinin kromozom yapıları, karyotip formülleri ve kromozom sayıları açısından incelenmesidir. Bu türlerin karyotipleri metafaz kromozomlarına bakılarak incelenmiştir. Sonuçlar üç türünde diploid (2n) kromozom sayısının 50 olduğunu göstermiştir. Catla catla, 22 akrosentrik, 2 subtelosentrik, 20 submetasentrik ve 6 metasentrik kromozom içerirken; Labeo rohita 32 akrosentrik, 4 subtelosentrik, 6 submetasentrik,8 metasentrik; Cirrhinus mrigla ise 30 akrosentrik, 8 subtelosentrik, 6 sub metasentrik ve 6 metasentrik kromozom içermektedir. Sentromerik index, (arm ratio) ve temel sayıda belirlenmiştir. Sitolojik olarak heteromorfik cinsiyete rastlanmamıştır. Bu üç türün kromozomlarının boyut, şekil ve (arm number) (NF) çeşitliliği aynı familya içerisindeki yapısal kromozom farklılıklarından ileri gelmektedir. Daha önceki çeşitli çalışmalar ile karşılaştırıldığında karyotip formüllerinde de varyasyonlara rastlanmıştır. Bunun nedeni olarak habitat özelliklerinde meydana gelen değişimlerin antropojenik aktivitelere etkisi düşünülebilir.

Anahtar Kelimeler: *Catla catla*, Chromosome, *Cirrhinus mrigla*, Karyotype, *Labeo rohita*

Introduction

Cyprinidae is a large family of freshwater fishes that is commonly called the carp family. The vast majority of bony fish belongs to this family and these are widely distributed in freshwater resources (Abdoli, 1999). Family Cyprinidae is comprised of 220 genera and 2420 species (Nelson, 2006). In fishes, cytogenetic data is important because in this group the usual morphotaxonomical characters are not so clear and phylogenetic understanding is beset with many hurdles. The increasing knowledge of chromosomes can provide reliable information on the phyletic relationship in the Cyprinidae to a certain extent (Kalbassi et al., 2006).

Systematically, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigla* belongs to class Teleostei, order Cypriniformes, family Cyprinidae (Jayaram, 1999). The study of fish chromosomes was initiated in India from 1960s by using basically the methodologies available for mammals. Karyological studies on Indian major carps have been carried out by some workers (Rishi, 1973; Khuda-Bukhsh and Manna, 1974; Majumdar and Ray-Chaudhari, 1976; Zhang and Reddy, 1991). The different variety of karyotypes found in different species was evidence that the process of evolution was associated with karyotypic changes (Fredga, 1977).

Chromosomal analysis is important for fish breeding from the viewpoint of genetic control, the rapid production of inbred lines, cytotaxonomy and evolutionary studies (Kirpichnikov, 1981). Karyological studies provide basic information on the number, size and morphology of chromosomes (Tan et al., 2004) that is important to undertake chromosome manipulation in fish (Khan et al., 2000). However, the studies dealing with karyotype of fishes are few because of large number and small size of chromosomes. Hence the present study was designed to determine the karyotype, chromosomal architecture, the proportion of acrocentric, submetacentric and metacentric chromosomes and number of chromosomes in Indian major carps, *C. catla, L. rohita, C. mrigla* from the geographic area of Haryana.

Materials and Methods

Fifteen fishes were obtained from the local fish farm and transported live to the Fish and Fisheries laboratory of Department of Zoology, Kurukshetra University, and Kurukshetra. Kidney and gill epithelium tissue were used for karyotype analysis. Three living specimens each of *C. catla*, *L. rohita* and *C. mrigla* from Haryana were analysed. The preparation of chromosomes was performed according to air drying technique given by Tjio and Whang (1965). Each specimen was injected intraperitoneally with a colchicine solution (0.05%; 1 mL/100g body weight). The fishes were maintained in a well aerated aquarium and after 2 hr., Kidney and gill epithelium were extracted and placed in hypotonic solution of 0.56% KCl. After 30 minutes in the hypotonic solution, the cellular suspension was centrifuged at 1000 rpm for 10 minutes. The hypotonic solution was discarded and the pellet was suspended and washed three times in methanol: glacial acetic acid (3: 1). After centrifugation at 1000 rpm

for 10 minutes, the drops of cellular suspension was put on a clean grease free microscopic slide, previously chilled in a freezer from a height of 2 feet. The slides were allowed to air dry.

For the conventional karyotype, the preparations were stained for 15 minutes with 5% Giemsa in phosphate buffer (pH 6.88). Mitotic metaphases spreads were scanned to determine the modal chromosome number. Mitotic metaphase were photographed by using Olympus C-7070 wide zoom camera at magnification of 1000X and used for preparation of karyotype. The arm ratio and centromeric indices of metaphase chromosomes were determined following Levan *et al*. (1964) to assign the morphological types and the chromosomal formulae.

Results and Discussion

Catla catla 2n = 50, NF^a = 76

The diploid chromosome number of all three major carps was found to be 50. In case of *C. catla*, the somatic metaphase showed the presence of 50 chromosomes (Figure 1A) and fundamental arm number 76. The somatic karyotype (Figure 1B) was prepared according to decreasing chromosome length. Somatic karyotype showed 22 acrocentric (pair nos. 4, 9, 11, 15-19, 22-23 and 25), 2 subtelocentric (pair no. 3), 20 submetacentric (pair nos. 1-2, 5-7, 12, 14, 20-21 and 24) and 6 metacentric (pair nos. 8, 10 and 13) chromosomes. Sex chromosomes could not be distinguished. The size of the chromosome ranged from as low as 0.251µm of 25th pair of chromosome to as high as 5.911µm of 1st pair of chromosomes (Table 1). Relative % length of the smallest chromosome was 0.640 while largest chromosome was 15.070. The total haploid mean length was calculated to be 39.218 µm.

Labeo rohita 2n = 50, NF^a = 64

The somatic metaphase in the kidney cells of the *L. rohita* showed diploid chromosome number 50 i. e. 2n=50 (Fig. 2A) and fundamental arm number 64. The somatic karyotype (Fig.2B) showed 32 acrocentric (pair nos. 2, 6-8, 10-13, 15, 18-21, 23-24), 4 subtelocentric (pair no. 4 and 14), 6 submetacentric (pair nos. 1, 9 and 16) and 8 metacentric (pair nos. 3, 5, 17 and 22) chromosomes. Sex chromosomes could not be distinguished. The size of the chromosomes in this case ranged from as low as 1.087 µm of 25th pair of chromosome to as high as 1.736 µm of 1st pair of chromosomes (Table 2). Relative % length of the smallest chromosome was 1.126 while largest chromosome was 9.528. The total haploid mean length was calculated to be 32.724 µm.

Cirrhinus mrigla 2n = 50, NF^a = 62

The somatic metaphase in *C. mrigla* also showed the presence of 50 chromosome number (Figure 3A) and fundamental arm number 62. The somatic karyotype (Figure 3B) comprised of 30 acrocentric (pair nos. 3, 5, 7, 9, 11-16, 19, 21-23 and 25), 8 subtelocentric (pair no. 2, 4, 6 and 20), 6 submetacentric (pair nos. 1, 10 and 24) and 6 metacentric (pair nos. 8, 17 and 18) chromosomes. Sex chromosomes could not be distinguished. The size of the chromosome ranged from 0.251 µm in case of 25th pair of chromosome and 1.400 µm of 1st pair of chromosomes (Table 3). Relative % length of the smallest chromosome was 3.357 while largest chromosome was 4.333. The total haploid mean length was calculated to be 29.575 µm.

In Cyprinidae 2n ranges from 44 to 100 (Arai, 1982). The high diploid chromosome number 2n=98-100 are thought to have resulted by polyploidisation of 2n=48 or 50. Chromosomal analysis in the present study revealed that these three Indian major carps from Haryana shared the same diploid number i. e. 2n=50. The karyological study of *C. catla* and *L. rohita* done by Khuda-Bukhsh and Manna (1974), Manna (1977), Majumdar and Ray Chaudhari (1976), Zhang and Reddy (1991), Jana (1993), Manna and Prasad (1971), Gui et al (1986) reported the similar results i. e. 2n=50. Karyotype studies on *C. mrigla* have been performed by Manna and Prasad (1971), Majumdar and Ray Chaudhari, (1976) and Zhang and Reddy (1991). All these studies have shown the diploid number as 50, confirming the present results. According to Manna (1984) and Rishi (1989) the most commonly occuring diploid number in family Cyprinidae is 50, considered to be the modal number of this species. Presence of same modal number in the present studies reinforces the hypothesis that Indian major carps are karyologically very conserved and represent plesiomorphic condition.

The primitive teleost karyotype is thought to have consisted of 46 to 48 chromosomes (Fitzsimons, 1972; LeGrande, 1975) all acrocentrics. Karyotypes with biarmed chromosomes are generally regarded to represent a derived condition (Fredga, 1977). Therefore, cyprinids investigated

in the present study showed a derived karyotype configuration. No heteromorphic sex chromosomes were found.

For comparative purpose, the arm number (NF) of karyotyped fishes is calculated assigning a value 2 to biarmed chromosomes (metacentric and submetacentric) and value of 1 to uniarmed chromosomes (acrocentric and subtelocentric) and is regarded as karyotype formulae. Despite the similarity of diploid numbers in all the three selected cyprinid species, there are differences in their karyotypic formulae. Comparision of the karyotypic formulae revealed deviations from the earlier reports for these species.

In case of *C. catla*, Khuda- Bukhsh and Manna (1976) reported 22 acrocentric, 24 submetacentric and 4 metacentric chromosomes. Manna (1977) reported 26 acrocentric, 16 submetacentric and 8 metacentric chromosomes. Zhang and Reddy (1991) and Jana (1993) showed similar results i. e. 22 acrocentric, 16 submetacentric and 12 metacentric chromosomes. But the present results showed a slight variation in chromosome morphology indicating 22 acrocentric, 2 subtelocentric, 20 submetacentric and 6 metacentric chromosomes.

Karyological study of *L. rohita* done by Manna and Prasad (1971), Majumdar and Ray Chaudhari (1976), Gui et al (1986), Zhang and Reddy (1991), Jana (1993) showed the same diploid number as found in present studies i.e. 2n=50. Manna and Prasad (1971) observed 24 subtelocentric, 8 submetacentric and 18 metacentric chromosomes. Gui et al (1986) observed 24 subtelocentric, 16 submetacentric and 10 metacentric chromosomes. Zhang and Reddy (1991) and Jana (1993) reported similar results in *L. rohita* i. e. 22 subtelocentric, 18 submetacentric and 10 metacentric chromosomes. Present results showed 32 acrocentric, 4 subtelocentric, 6 submetacentric and 8 metacentric chromosomes.

In case of *C. mrigla*, different chromosome morphology was given by different workers but basic diploid number was same i.e. 2n=50. Manna and Prasad (1971) reported 36 acrocentric, 8 submetacentric and 6 metacentric chromosomes which showed little similarity with the present results. In the present study 30 acrocentric, 6 submetacentric, 6 metacentric and 8 subtelocentric chromosomes were found. Majumdar and Ray Chaudhari (1976) observed 18 acrocentric, 26 submetacentric and 6 metacentric chromosomes. Zhang and Reddy (1991) showed 20 acrocentric, 18 submetacentric and 12 metacentric chromosomes. Many authors considered that diploid chromosomes are all acrocentric as the ancestral karyotype in fishes (Nogusa, 1960; Post, 1965; Denton, 1973). It may be pointed out that all acrocentric karyotype happens to be absent in a number of primitive group like chondrichthyes. Therefore it may be assumed that acrocentricity is certainily a more primitive condition that the biarmed condition.

The acrocentric chromosomes have a tendency to stick to each other by their centromere and in this way they form metacentric chromosomes (Dogramci et al. 1994). Denton (1973), Gold (1979) also stated that karyotypes with biarmed chromosomes are regarded as derived condition confirming that karyomorphology of all the three species could be derived mainly by envisaging per centric inversion at various regions with respect to time, geographical condition and ecological characteristics.

A comparision of karyotypic formulae of these three Indian major carps species revealed that larger numbers of acrocentric chromosome are observed in *L. rohita* followed by *C. mrigla* and *C. catla.* 6 metacentric chromosomes were observed in both *C. mrigla* and *C. catla* whereas 8 metacentric chromosomes were observed in *L. rohita*. According to Le Grande (1981), differences in the NF among close species can be the result of pericentric inversions. The karyotype formulae of Indian major carps in the present studies can be interpreted as the result of structural chromosomal rearrangements as well as a series of pericentric inversions, generating biarmed chromosomes and so increase the NF to 74 in *C. catla*, 64 in *L. rohita* and 62 in *C. mrigla.* The karyotypes of these species have been compared with the related ones and it has been suggested that large number of acrocentric chromosomes have been observed during present investigation in comparision to earlier studies. It may be due to mechanism of centric fission. Centric fission seemed to have played a significant role in evolution of teleost fishes (Manna and Khuda-Bukhsh, 1978). Different groups of fishes exhibit different processes of karyotype evolution.

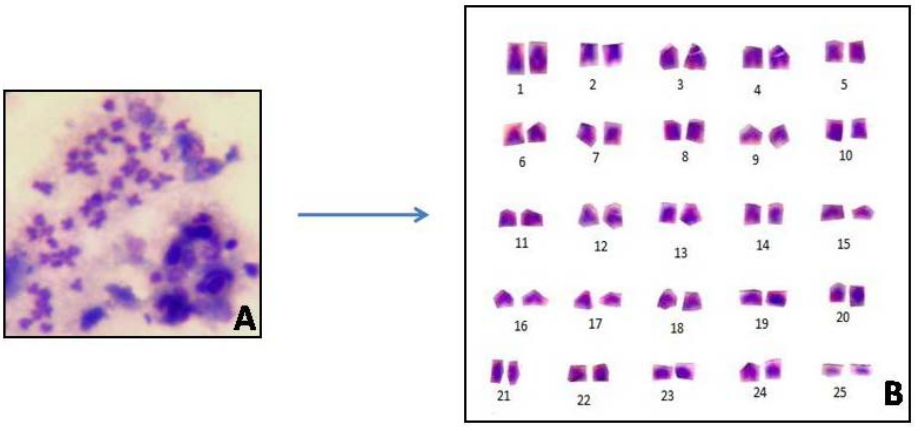

Fig. 1

Figure 1A. Somatic metaphase of *C. catla*
Figure 1B. Karyotype of *C. catla*

Table 1. Morphometric data of somatic karyotype of *C. catla* (2n= 50)

Chrm. Pair no.	Short arm length (µm) (p)	Long arm length (µm) (q)	Total length (µm)	% Relative length (RL%)	Arm ratio	Centromeric Index (%)	Chromosome morphology
1	1.960	3.951	5.911	15.072	2.015	33.158	Submetacentric
2	1.981	3.661	5.642	14.386	1.848	35.111	Submetacentric
3	1.020	3.540	4.560	11.627	3.470	22.368	Subtelocentric
4		3.520	3.520	8.975			Acrocentric
5	0.998	1.603	2.601	6.632	1.606	38.369	Submetacentric
6	0.781	1.413	2.194	5.594	1.809	35.597	Submetacentric
7	0.615	1.054	1.669	4.255	1.713	36.848	Submetacentric
8	0.584	0.852	1.436	3.661	1.458	40.668	Metacentric
9		1.210	1.210	3.085			Acrocentric
10	0.480	0.715	1.195	3.047	1.489	67.132	Metacentric
11		1.091	1.091	2.781			Acrocentric
12	0.385	0.684	1.069	2.725	1.776	56.286	Submetacentric
13	0.384	0.551	0.935	2.384	1.434	69.691	Metacentric
14	0.354	0.541	0.895	2.282	1.528	65.434	Submetacentric
15		0.715	0.715	1.823			Acrocentric
16		0.691	0.691	1.761			Acrocentric
17		0.598	0.598	1.524			Acrocentric
18		0.544	0.544	1.387			Acrocentric
19		0.523	0.523	1.333			Acrocentric
20	0.158	0.301	0.459	1.170	1.898	34.422	Submetacentric
21	0.145	0.285	0.430	1.096	1.965	33.720	Submetacentric
22		0.398	0.398	1.014			Acrocentric
23		0.377	0.377	0.961			Acrocentric
24	0.115	0.189	0.304	0.775	1.643	37.828	Submetacentric
25		0.251	0.251	0.640			Acrocentric

Total mean haploid length = 39.218 µm

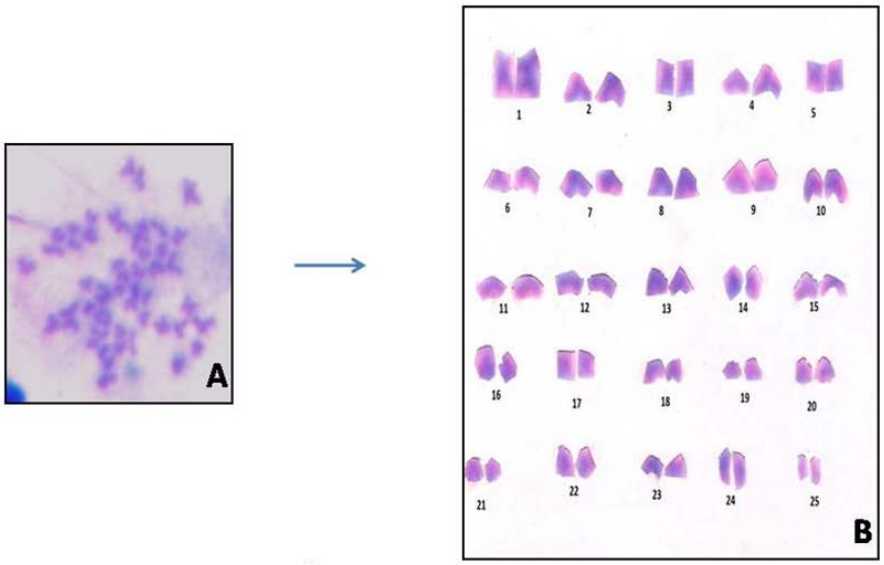

Fig. 2

Figure 2A. Somatic metaphase of *L. rohita*
Figure 2B. Karyotype of *L. rohita*

Table 2. Morphometric data of somatic karyotype of *L. rohita* (2n= 50)

Chrm. Pair no.	Short arm length (µm) (p)	Long arm length (µm) (q)	Total length (µm)	% Relative length (RL%)	Arm ratio	Centromeric Index (%)	Chromosome morphology
1	0.756	0.980	1.736	9.528	1.875	34.782	Submetacentric
2		1.543	1.543	7.030			Acrocentric
3	0.681	0.784	1.465	6.020	1.569	38.924	Metacentric
4	0.587	0.874	1.461	5.968	4.298	23.262	Subtelocentric
5	0.719	0.734	1.453	5.864	1.068	48.344	Metacentric
6		1.379	1.379	4.906			Acrocentric
7		1.373	1.373	4.829			Acrocentric
8		1.361	1.361	4.673			Acrocentric
9	0.601	0.759	1.360	4.660	2.564	28.055	Submetacentric
10		1.343	1.343	4.440			Acrocentric
11		1.341	1.341	4.418			Acrocentric
12		1.337	1.337	4.363			Acrocentric
13		1.329	1.329	4.259			Acrocentric
14	0.591	0.701	1.292	3.780	3.197	31.164	Subtelocentric
15		1.281	1.281	3.638			Acrocentric
16	0.587	0.692	1.279	3.612	2.206	31.182	Submetacentric
17	0.598	0.601	1.199	2.576	1.030	49.246	Metacentric
18		1.187	1.187	2.421			Acrocentric
19		1.178	1.178	2.304			Acrocentric
20		1.174	1.174	2.252			Acrocentric
21		1.168	1.168	2.175			Acrocentric
22	0.568	0.580	1.159	2.058	0.176	42.767	Metacentric
23		1.140	1.140	1.812			Acrocentric
24		1.099	1.099	1.281			Acrocentric
25		1.087	1.087	1.126			Acrocentric

Total mean haploid length = 32.724 µm

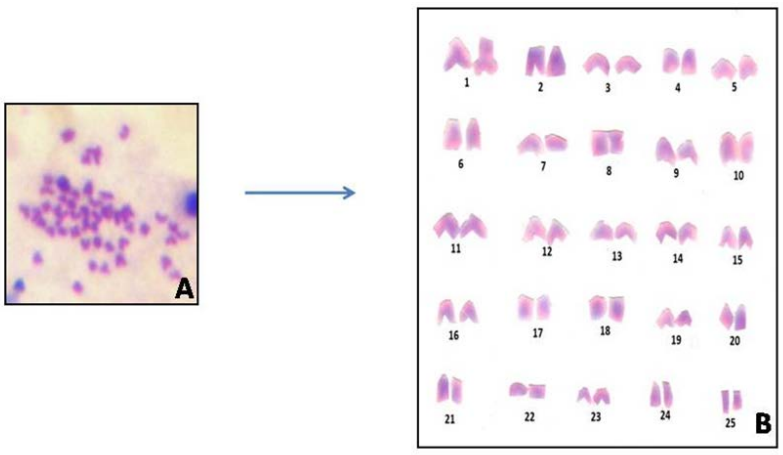

Fig. 3

Figure 3A. Somatic metaphase of *C. mrigla*

Figure 3B. Karyotype of *C. mrigla*

Table 3. Morphometric data of somatic karyotype of *C. mrigla* (2n= 50)

Chrm. Pair no.	Short arm length (µm) (p)	Long arm length (µm) (q)	Total length (µm)	% Relative length (RL%)	Arm ratio	Centromeric Index (%)	Chromosome morphology
1	0.499	0.901	1.400	4.733	1.805	35.642	Submetacentric
2	0.300	1.099	1.399	4.730	3.651	21.443	Subtelocentric
3		1.395	1.395	4.716			Acrocentric
4	0.291	1.096	1.387	4.689	3.766	20.980	Subtelocentric
5		1.381	1.381	4.669			Acrocentric
6	0.288	1.089	1.377	4.655	3.781	20.915	Subtelocentric
7		1.375	1.375	4.649			Acrocentric
8	0.683	0.691	1.374	4.645	1.011	49.708	Metacentric
9		1.374	1.374	4.645			Acrocentric
10	0.299	1.073	1.372	4.639	3.588	21.793	Submetacentric
11		1.370	1.370	4.632			Acrocentric
12		1.369	1.369	4.628			Acrocentric
13		1.368	1.368	4.625			Acrocentric
14		1.365	1.365	4.615			Acrocentric
15		1.361	1.361	4.601			Acrocentric
16		1.358	1.358	4.591			Acrocentric
17	0.651	0.705	1.356	4.581	1.082	48.008	Metacentric
18	0.651	0.702	1.353	4.574	1.078	48.115	Metacentric
19		1.347	1.347	4.554			Acrocentric
20	0.277	1.067	1.344	4.554	3.851	20.610	Subtelocentric
21		1.399	1.399	4.527			Acrocentric
22		1.398	1.398	4.527			Acrocentric
23		1.374	1.374	4.645			Acrocentric
24	0.275	0.925	0.304	4.057	3.363	20.916	Submetacentric
25		0.251	0.251	3.357			Acrocentric

Total mean haploid length = 29.575 µm

Conclusions

In conclusion, the chromosome analysis of three Indian major carp species *C. catla* (Hamilton, 1822), *L. rohita* (Hamilton, 1822), *C. mrigla* (Hamilton,1822) using conventional staining procedure revealed the same diploid number (2n=50) with variability in size, shape and arm number (NF) of chromosomes suggesting that diversification in these fishes of the same family is related to structural changes in chromosomes. The variations in karyotype formulae in comparison to earlier studies may be because of pericentric inversions or centric fission and appears to be due to variations in habitat conditions as a result of anthropogenic activities.

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

SHORT COMMUNICATION KISA MAKALE

MELET IRMAĞI'NDA (ORDU) *Cladophora crispata*'da BAZI AĞIR METAL DÜZEYLERİ

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Öz: Evsel ve endüstriyel atıklar ile kontamine olmuş alanlardaki yüksek besin düzeyi *Cladophora* türlerinin yayılımını artırmaktadır. Bu türler genellikle ağır metal ile kontamine olmuş sucul çevredeki en iyi biyoindikatör olarak bilinmektedir. Bu çalışmada, Ordu ilinin içme suyunun teminin edildiği önemli bir akarsu olan Melet Irmağı üzerindeki istasyonlardan toplanan *Cladophora crispata* örneklerindeki kadmiyum, kobalt, krom, bakır, kurşun, nikel, demir ve çinko konsantrasyonlarının belirlenmesi amaçlanmıştır. Bu amaçla belirlenen dört farklı istasyondan toplanan örnekler endüktif eşleşmiş plazma-kütle spektrometrisi (ICP-MS) ve atomik absorbsiyon spektrofotometre (AAS)'de analiz edilmiştir. Ağır metallerin ölçüm değerleri istatistiksel olarak değerlendirilmiştir. Bakır, kurşun ve çinko maden işletmesine yakın ikinci istasyondaki *Cladophora crispata* örneklerinde kurşun (Pb; 844,9 µg/g) değerleri diğer istasyonlara ait ölçüm değerleriyle karşılaştırıldığında (ANOVA), farklar anlamlı ve önemli bulunmuştur. Bu çalışma Melet Irmağı'nda ağır metal konusunda yapılan ilk çalışmadır.

Anahtar Kelimeler: *Cladophora crispata*, Ağır metal birikimi, ICP-MS, Melet Irmağı

Abstract: **Heavy Metal Levels in *Cladophora crispata* in Melet River (Ordu)**

Cladophora species accumulate high nutrient levels in the areas contaminated by domestic and industrial wastes. This species are generally considered as the best bioindicator of aquatic ecosystem contamination by heavy metals. The object of this study was to investigate to cadmium, cobalt, chrome, copper, lead, nickel, iron and zinc concentration of *Cladophora crispata* samples taken from stations on Melet River provided drinking water in Ordu. For this aim, the concentrations of heavy metals in *Cladophora crispata* sample were collected from four different stations, were determined using ICP-MS and AAS methods. All heavy metals were statistically analysed and evaluated. When the lead (Pb; 844.9 µg/g) values of the *Cladophora crispata* samples collected from the second stations near copper, lead and zinc mining industry were compared with the data from other stations (ANOVA), the differences were meaningful and significant.

Keywords: *Cladophora crispata*, Heavy metal accumulation, ICP-MS, Melet River.

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Giriş

Ülkemizde son yıllarda giderek artan çevresel problemlerin başında ağır metal iyonlarından kaynaklanan su kirliliği gelmektedir. Su kirliliğinin artması endüstri alanındaki büyümeyi çok iyi bir şekilde yansıtmaktadır. Endüstriyel işlem ve ürünlerde ağır metal kullanımı son yıllarda hızla artmakta ve buna bağlı olarak sucul ortamda yaşayan hayvansal ve bitkisel canlılar üzerinde birçok olumsuzluğa sebep olmaktadır (Foy ve ark., 1978, Kayhan ve ark., 2009).

Bulundukları su ortamı ve diğer canlılar ile sürekli etkileşimde olan akuatik organizmalardaki kirlilik ortamı potansiyel kirlilik seviyesini temsil etmektedir (Taylan ve Özkoç, 2007). Özellikle mikroorganizma grubu içerisinde algler de dahil olmak üzere birçok bakteri ve mantar türü metal kirliliğinin dağılımı ve birikimini araştırmak için kullanılmaktadır (Sağlam, 1995). Bunun yanında; su örnekleri, sedimentler, sucul bitkiler, gastropodlar ve balıklar da kirliliğin tespiti için kullanılmaktadır (Dallinger, 1994, Rai ve ark., 1995, Canlı ve Atlı, 2003, Elmacı ve ark., 2005, Arıman ve ark., 2007).

Akuatik sistemde ekolojik açıdan önemli bir grup olan Chlorophyta içinde yer alan makro algler birçok araştırmacı tarafından ağır metal kirliliğinin göstergesi (indikatörü) olarak kullanılmaktadır (McCormick ve Cairns, 1994). En yaygın olarak kullanılanlar *Enteromorpha* (Bat ve ark., 2001, Villares ve ark., 2001), *Ulva* (Bat ve ark., 2001, Tüzen, 2002, Boubonari ve ark., 2008, Kamala-Kannan ve ark., 2008), *Cladophora* (Vymazal, 1989, Oertel, 1991, Chmielewska ve Medved, 2001, Çavuşoğlu ve ark., 2007) ve *Hormidium* (Rai ve ark., 2008) türleridir. *Cladophora* türleri sucul ortamdaki ağır metal kirliliğinin araştırılmasında kullanılan en iyi biyoindikatörler arasında yer almaktadır (Whitton ve ark., 1989, Oertel, 1991, Graham ve Wilcox, 2000, Çavuşoğlu ve ark., 2007). 1976'dan günümüze ortamdaki çeşitli ağır metal kaynaklı kirliliklerin saptanmasında *Cladophora* örnekleri sıklıkla kullanılmaktadır (Keeney ve ark., 1976; Vymazal, 1984; Vymazal, 1987; Whitton ve ark., 1989; Vymazal, 1989; McHardy ve George, 1990; Oertel, 1991; Chmielewska ve Medved, 2001; Çavuşoğlu ve ark., 2007; Deng ve ark, 2009; Atıcı ve ark., 2010). *Cladophora crispata* kullanılarak krom, nikel, kadmiyum ve çinko ağır metallerinin giderimine yönelik çalışmaların sayısı artmaktadır (Aksu ve ark., 1996; Özer ve ark., 1999; Özer ve Özer, 1998; Özer ve ark., 2000).

Bu çalışmada, Ordu ilinin içme suyunun karşılandığı, evsel, tarımsal, maden işletmeciliği ve doğal maden rezervlerinden kaynaklı kirlenen Melet Irmağı'ndaki metal kirliliğinin sucul ekosistemdeki etkisi, besin zincirinin ilk halkasını oluşturan ve ağır metal birikimi açısından sucul sistemdeki en iyi biyoindikatör türlerden biri olan *Cladophora crispata* kullanılarak incelenmesi amaçlanmıştır. Bu amaçla Melet Irmağı üzerinde ırmak sucul ekosistemine etki edebilecek yerler göz önünde bulundurularak belirlenen dört istasyondan toplanan *Cladophora crispata* örneklerindeki kadmiyum, krom, kobalt, bakır, kurşun, nikel, demir ve çinko birikimleri incelenmiştir.

Materyal ve Metot

Melet Irmağı'nda 2008 yılının Temmuz ayında yoğun popülasyon oluşturan *Cladophora crispata* örnekleri ve su örnekleri belirlenen 4 istasyondan toplandı. Toplanan alg örneklerdeki ağır metal (Cd, Cr, Co, Cu, Pb, Ni, Fe ve Zn) konsantrasyonları belirlenmiştir. Örnekleme noktalarında çözünmüş oksijen, pH, sıcaklık ve çözünmüş oksijen gibi fiziksel parametrelerin ölçümü taşınabilir sistem kullanılarak gerçekleştirilmiştir.

Çalışma Alanının Tanımı

Çalışma alanını oluşturan Melet Irmağı, Ordu ilinin en önemli akarsuyu olup bölgenin Orta ve Doğu Karadeniz bölümleri arasında doğal bir sınır oluşturmaktadır. Melet Irmağı 3167 m yüksekliğindeki Karagöl Dağları üzerinde doğarak Mesudiye'yi 3 km geçtikten sonra Esat Deresi'ni de alıp kuzeye yönelir, buradan sonra irili ufaklı derelerle birleşerek yaklaşık 85 km sonra Ordu ilinin doğusunda belirgin olmayan bir delta oluşturarak Karadeniz'e dökülmektedir (DSİ, 2003). Ordu ilinin içme suyunun yaklaşık %60'ı Melet Irmağı'ndan kar-

şılanmaktadır. Bu nedenle ırmak kent için önemli bir tatlı su rezervidir.

Bu çalışmada, ağır metal analizleri için kullanılacak olan *Cladophora crispata* örnekleri Melet Irmağı üzerinde belirlenen istasyonlardan toplanmıştır. Maden ocağı işletmelerinin yakını, fındık tarımının yapıldığı yerler vb. ırmak sucul ekosistemine etki edebilecek yerler göz önünde bulundurularak belirlenen 4 istasyonun konumları Şekil 1'de verilmiştir. Numune alınan birinci istasyon Güzelyurt Köyü, Koyulhisar (Sivas), ikinci istasyon Kızılelma Köyü, Koyulhisar (Sivas), üçüncü istasyon Mesudiye (Ordu), dördüncü istasyon Bayadı Köyü, Merkez (Ordu) olarak belirlenmiştir.

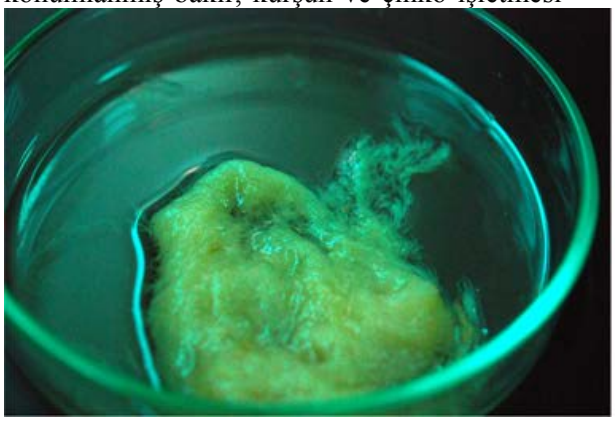
Birinci istasyonun (Güzelyurt Köyü-Koyulhisar) güneydoğusunda Kurşunlu mevkiinde bakır, kurşun ve çinko cevher damarları yer almaktadır (Gökçe, 1990). Örneklememizin yapıldığı Devren Deresi de Çandır mevkiinde yer alan bakır, kurşun ve çinko üretimi yapan bir maden işletmesine yakın geçmekte ve buradan da Melet Imağı'na karışmaktadır. Buna ek olarak Sivas ili Gümüşlü ve Kurşunlu mevkii ve çevresinde krom ve gümüş çıkarımı için birçok işletme yer almaktadır. Bu işletmelerin de yakınlarından geçen birçok dere bulunmakta ve bu dereler de Melet Irmağı'na karışmaktadır.

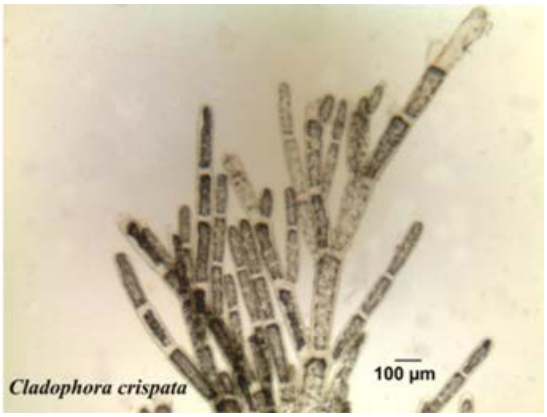
Birinci istasyon doğal maden rezervlerine yakın buralardaki işletmelere uzak bir yer seçilirken, ikinci istasyon (Kızılelma Köyü-Koyulhisar) özellikle bakır, kurşun ve çinko işletmesi yapan maden işletmesi güzergahında seçilmiştir. Üçüncü istasyon (Mesudiye-Ordu) diğer istasyonlarla karşılaştırıldığında yerleşim alanına en yakın istasyondur. Dördüncü istasyonun bulunduğu bölgede Kabadüz ilçesi Akgüney Köyü'nde Melet Irmağı Havzası'nda konumlanmış bakır, kurşun ve çinko işletmesi bulunmaktadır. Örneklememizin yapıldığı 2008 tarihinde devir işlemlerinden dolayı maden işletmesi kapalıdır. Fakat işletme sahasında önceki çalışmalardan dolayı depolanmış atık maddeler açık havuzlarda bulunmaktadır.

Şekil 1. Çalışma istasyonlarını gösteren harita (Google Earth programı kullanılmış ve modifiye edilmiştir).

Cladophora crispata

Cladophora crispata, silindirik hücrelerden oluşmakta ve düzensiz dallanma göstermektedir. Ana kol uç hücre uzunluğu 40-75 µm, yan kollardaki uç hücre uzunluğu ise 20-35 µm olup uç hücreler hafif bir şekilde sivrilme göstermektedir (Prescott, 1970, John, 2002). *Cladophora crispata*'ya ait görünümler Şekil 2'de verilmiştir.





Şekil 2. *Cladophora crispata* örneğinin makro (solda) ve mikroskobik görüntüsü (sağda).

Alg Örneklerinin Analizi

C. crispata örneklerinden 1g alınarak ısıya dayanıklı cam şişeler içerisine bırakılmış ve kurutma fırınında 105°C de 24 saat kurutulmuştur.

Endüktif Eşleşmiş Plazma-Kütle Spektrometrisi (ICP-MS) Analizi

Homojenize edilen yaklaşık 0.5g numunelerden tam tartım alınarak, nitrik asit, hidroklorik asit, hidrojenperoksit ve hidroflorik asit karışımı ile Anton Paar 3000 Multiwave mikrodalga fırında uygun sıcaklık/basınç programı uygulanarak çözme işlemi tamamlandı. Mikrodalga çözme sistemi ile hazırlanan çözeltilerdeki Cr, Ni, Co, Cu, Cd, ve Pb (toplam) miktarları Agilent 7500a ICP-MS ile ölçüldü (Chmielewska ve Medved, 2001).

Atomik Absorbsiyon Spektrofotometresi (AAS) Analizi

Beklenen derişim düzeylerinin yüksek olması nedeniyle Fe ve Zn elementlerinin tayini için Perkin Elmer Analyst 800 F-AAS cihazı kullanıldı (Chmielewska ve Medved, 2001). Tüm bu işlemler Tübitak Ankara Test ve Analiz Laboratuvarı'nda yapıldı.

İstatistiksel Analiz

Cladophora crispata öreklerindeki ağır metal birikim düzeylerinin istasyonlar arasındaki farklılıklarının tespiti tek yönlü varyans analizi (ANOVA) ve Duncan's testi ile gerçekleştirildi.

Bulgular ve Tartışma

Bu çalışmada Ordu İli içme suyunun karşılandığı Melet Irmağı kıyılarında Temmuz ayında yoğun popülasyonlar oluşturan *C. crispata*'nın absorbe ettiği ağır metal yükleri araştırılmıştır. Farklı istasyonlardan toplanan örneklerdeki kadmiyum (Cd), kobalt (Co), krom (Cr), bakır (Cu), kurşun (Pb), nikel (Ni), demir (Fe) ve çinko (Zn) düzeyleri araştırılmış ve elde edilen sonuçlar değerlendirilmiştir.

Örnekleme istasyonlarında, alınan alg örneklerindeki metal konsantrasyonları ile muhtemel ilişkileri bulabilmek için sudaki fizikokimyasal özellikler saptanmıştır. İstasyonlarda ölçülen sıcaklık (21.9-26.7) ve pH (7.77-9.21) değerlerinde birinci istasyondan dördüncü istasyona doğru artış görülmüştür. Qertel (1993) tarafından Danube Nehri üzerinde *Cladophora glomerata* kullanılarak yapılan çalışmada sel, ışık, sıcaklık, iletkenlik ve redoks potansiyeli gibi fiziksel ve kimyasal parametrelerin dolaylı olarak ağır metal alınımı ve birikiminde etkili en önemli faktörler olduğunu göstermiştir. Bu durum bize ikinci istasyonun ağır metal yükünü göz önünde bulundurduğumuzda metallerden kaynaklı kirliliğin buradaki canlılara toksik etkisinin çok daha fazla olabileceğini göstermektedir.

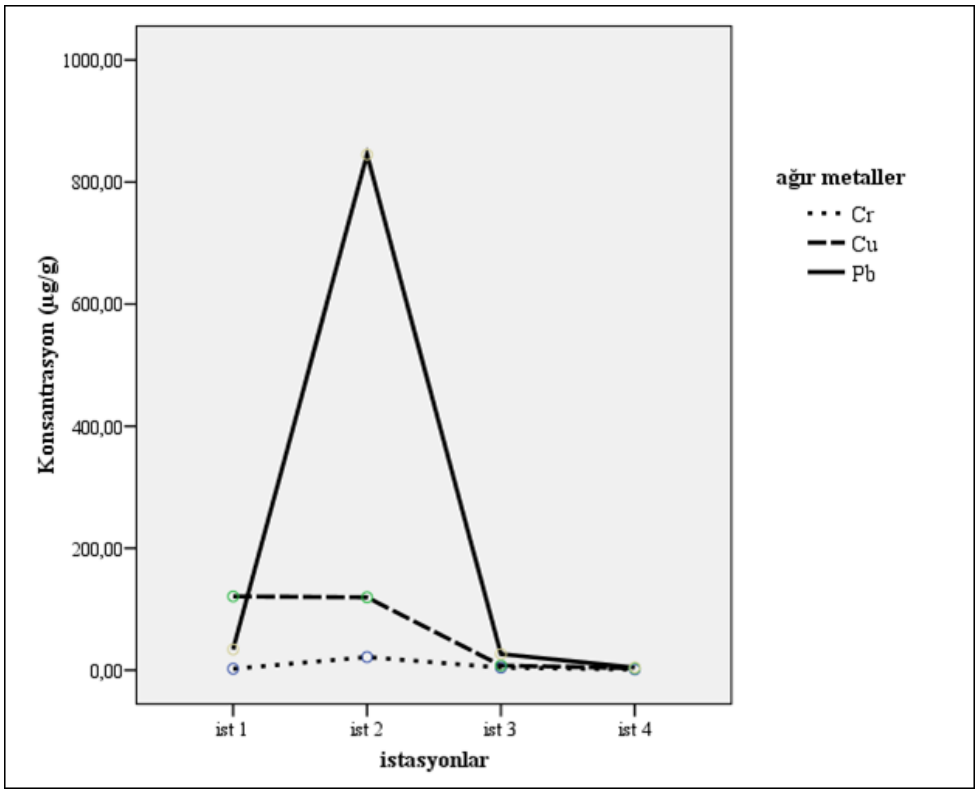
Tüm istasyonlardan alınan *C. crispata* örneklerine ait ağır metal konsantrasyonları Tablo 1'de verilmiştir. İstasyonlardan alınan alg örnekleri metal miktarları açısından yapılan istatistiksel değerlendirmede istasyonlar arasında yalnızca kurşun yönünden önemli bir fark ($p<0.05$) gözlendi. Duncan's testi sonucuna göre ağır metallerin önem sırası; Pb > Cu > Cr > Co > Ni > Cd > Fe > Zn, istasyonlar arası önem sırası ise; İst2 > İst1 > İst3 > İst 4 şeklindedir.

Bakır, kurşun ve çinko maden rezervine sahip olan birinci istasyon ağır metal birikimi yönünden yoğun bir istasyondur. Bu elementlerden bakır, kurşun, kobalt ve nikel oranlarının yüksek olduğu görülmektedir. Birinci istasyonun güneyine yakın Kurşunlu Mevkii ile Melet Irmağı arasında Cu, Pb ve Zn cevher damarları yer almaktadır (Gökçe, 1990). Hem bakır, kurşun ve çinko maden rezervine sahip hem de çeşitli maden işletmelerine ev sahipliği yapan ikinci istasyon, dört istasyon içerisinde *C. crispata*'da ağır metal birikimi yönünden en yoğun istasyondur. Bu istasyondan toplanan alg örneklerinde kurşun (844.9 µg/g), bakır (119.5 µg/g), krom (21.66 µg/g), kadmiyum (5.135 µg/g) ve çinko (1.5937 µg/g) ağır metallerinin oranlarının diğer istasyonlara göre oldukça yüksek olduğu görülmektedir.

Birinci istasyonda örneklemenin yapıldığı Devren Deresi bu bölgede yer alan bakır-çinko-kurşun işletmesine oldukça yakın geçmekte ve buradan da Melet Irmağı'na karışmaktadır. Buna ek olarak Sivas ili Gümüşlü ve Kurşunlu Mevkii ve çevresinde adlarından da anlaşılacağı üzere bakır, kurşun ve çinko ele-

mentlerinin yanı sıra krom ve gümüş çıkarımı için birçok işletme bulunmaktadır. Birinci ve ikinci istasyonun bulunduğu bu alandaki işletmelerin yakınlarından geçen dereler Melet Irmağı'na karışmaktadır. Bu durum çalışmamızın sonucunda birinci istasyonda Co (20.29 µg/g), Cu (121.0 µg/g) ve ikinci istasyonda, Cu (119.5 µg/g), Pb (844,9 µg/g), Cr (21.66 µg/g), Cd (5.135 µg/g) ve Zn (1.5937 µg/g) değerlerinin yüksek çıkmasını açıklamaktadır. Şekil 3'teki interaksiyon grafiğinde de görüldüğü üzere her iki istasyondan elde edilen sonuçlar, ırmağın bu istasyonlardan önce hem doğal hem de işletme kaynaklı kirlenmeye maruz kaldığını göstermektedir.

Üçüncü istasyondaki ağır metal birikiminin boyutları dördüncü istasyondaki ile hemen hemen aynıdır ve diğer istasyonlara göre ağır metal birikimi açısından en düşük yoğunluğa sahip istasyonlardır. Dördüncü istasyonun yakınında başka bir Pb-Cu-Zn maden işletmesi bulunmasına rağmen örneklemenin yapıldığı 2008 tarihinde bu işletmenin kapalı olduğu bilinmektedir. Bundan dolayı her iki istasyondaki birikimin düşük olmasının nedeni olarak doğal maden rezervinin diğer istasyonlara göre oldukça düşük oluşu, sedimentteki birikimden dolayı aşağı havzaya kadar birikimdeki azalma ve her iki istasyonun da bu maden işletmesinden oldukça uzakta bulunması olarak verilebilmektedir.



Şekil 3. İstasyonlara göre ağır metal konsantrasyonlarını gösteren interaksiyon grafiği.

Tablo 1. *C. crispata* örneklerindeki ağır metal konsantrasyonlarının istasyonlara göre ortalama ±ss değerleri (µg g^{-1} kuru ağırlık, n=3) ve tanımlayıcı istatistik değerleri

Ağır metaller		Cd	Co	Cr	Cu	**Pb**	Ni	Fe	Zn
İstasyonlar	İst 1	2.7 ± 0.05	20.3 ± 0.28	2.5 ± 0.05	121.0 ± 11.13	34.2 ± 0.29	7.5 ± 0.10	0.3 ± 0.03	1,1 ± 0,12
	İst 2	5.1 ± 0.13	4.4 ± 0.18	21.7 ± 0.35	119.5 ± 2.21	844.9 ± 5.81	3.8 ± 0.14	0.3 ± 0.03	1,6 ± 0,06
	İst 3	0.5 ± 0.03	2.0 ± 0.05	4.4 ± 0.04	7.7 ± 0.34	26.6 ± 1.30	2.6 ± 0.06	2.2 ± 0.10	0,3 ± 0,04
	İst 4	3.9 ± 0.09	0.9 ± 0.32	1.4 ± 0.01	3.4 ± 0.10	4.5 ± 0.19	2.8 ± 0.32	2.5 ± 0.62	0,2 ± 0,04
En düşük		0,50	0.56	1.36	3.26	4.31	2.48	0.26	0.21
En yüksek		5,27	20.57	22.01	132.13	850.71	7.62	3.15	1.65
Veri sayısı (n)		12	12	12	12	12	12	12	12
Ortalama		3,05	6.90	7.48	62.90	227.53	4.19	1.32	0.81
Standart sapma		1,77	8.18	8.63	60.12	372.47	2.06	1.11	0.58
p*		0,88	0.09	0.05	0.17	**0.02***	0.24	0.20	0.35

* $p<0.05$ önemli

Birinci ve ikinci istasyon alanlarının Cu, Pb ve Zn açısından doğal bir rezerv olduğunu göz önüne alırsak ortamdaki bu iki istasyondaki elementler karşılaştırıldığında kadmiyum, bakır, nikel, demir ve çinko miktarlarında ciddi bir farklılık göze çarpmamaktadır. Fakat ikinci istasyondaki kurşun birikiminin (844.9 µg/g) birinci istasyona (34.160 µg/g) göre yaklaşık 25 kat fazla oluşu ve bunun yanında krom miktarındaki artış bölgenin bu elementlere ev sahipliği yapmasının yanı sıra akarsuya bir karışımın olduğunu göstermektedir.

Çavuşoğlu ve ark. (2007) tarafından yapılan çalışma ile karşılaştırıldığında Kızılırmak Nehri çevresindeki sanayi kuruluşları yakınındaki ikinci istasyondan toplanan *Cladophora* örneklerinde başta kurşun olmak üzere yüksek miktarlarda krom, bakır, çinko ve kadmiyuma rastlanmıştır. Çalışmamızın yapıldığı Melet Irmağı'nda da maden işletmesinin yakınında bulunan ikinci istasyonda kurşun, bakır, krom, kadmiyum ve çinko oldukça yüksek değerlerde bulunmuştur. Her iki çalışmada da işletmelere veya sanayi kuruluşlarına yakın olan istasyonlarda ve benzer metallerin yüksek bulunması açısından paralellik göstermektedir.

Sonuç

Çalışmamızda elde edilen bulgular ikinci ve üçüncü istasyonlarda bulunan öncelikle kurşun, devamında bakır, krom ve demir metallerindeki yüksek birikim bu istasyonların çevresinde yerleşim yeri bulunmasından dolayı tehlikeli boyutlarda olduğunu göstermektedir. Bilindiği gibi çevrede birçok organizma kimyasal maddelere karşı oldukça duyarlılık göstermektedir. Maden işletmelerine ve yerleşim yerine yakın olan dereler vasıtasıyla ağır metal, evsel ve tarımsal atıkların Melet Irmağı'na ve oradan da Karadeniz'e karışması ırmak ve denizel ekosistemdeki her bir bireyin biyolojik aktivitesini olumsuz yönde etkileyecektir.

Melet Irmağı'ndaki ağır metal kirliliğinin dördüncü istasyonda çok tehlikeli boyutlarda olmadığını göstermiştir. Dördüncü istasyonun bulunduğu, Kabadüz ilçesi Bakacak mevkiindeki Cu, Pb ve Zn maden işletmesinin örneklemenin yapıldığı Temmuz 2008'de kapalı durumdadır. 29 Temmuz 2009 tarihinde bu maden işletmesinden çıkan atık maddelerin bulunduğu havuzlar sel nedeniyle çökmüş ve Ordu ilinin içme suyunun sağlandığı Melet Irmağı kirlenmiştir. Faaliyette olmayan maden tesisinde 23 Eylül 2009'da ise yaşanan sel felaketi etkisiyle pasa havuzunun üst seviyesine kadar dolan yağmur suyu, daha önce işletme sürecinde depolanan artık maddenin bir kısmı ile birlikte içme suyunun karşılandığı Melet Irmağı'na karışmıştır. Yerel ve ulusal basında yaşanan bu olayın nasıl gerçekleştiği ve işletme kaynaklı ırmağın nasıl kirletildiğine büyük ölçüde yer verilmiştir. Bunun yanında İl Çevre ve Orman Müdürlüğü ekipleri tarafından su numunesi alınarak Samsun Bölge Hıfzısıhha Müdürlüğü'ne gönderilerek yapılan analizler sonucu sudaki kurşun miktarı 338 µg/L'ye kadar yükseldiği tespit edilmiştir. Su Kalite Kontrol Yönetmeliği'ne göre içilebilir nitelikteki 1. kalite suyun içerisindeki Pb miktarının 10 µg/L olması gerekmektedir (SKKY, 2008).

Çalışmamızdan elde edilen bulgular ışığında, Melet Irmağı'ndaki kirlilik düzeyini belirlemek ve takibi için su, sediment ve biyomonitör organizmalar seçilerek kapsamlı ve sürekli bir çalışma yürütülmesi önerilmektedir. Bunun için besin zincirindeki birçok organizma baz alınarak yapılacak olan toksisite çalışmaları zincirin en üst kademesindeki insanda oluşabilecek birikimleri ve etkileri belirlememiz ve yorumlamamızı sağlayacaktır. Bu ve buna benzer çalışmalar belirli aralıklarla tekrarlandığında su kirliliğinin ulaştığı boyutlar hakkında bilgi sahibi olarak Ordu ili içme suyu kaynağı olarak büyük öneme sahip Melet Irmağı'ndaki kirliliğin asıl sebepleri ortaya konulacak ve çalışmaların tekrarlanması ile de çevredeki kuruluşların denetimleri sağlanacaktır.

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SHORT COMMUNICATION KISA MAKALE

INVESTIGATION OF CERTAIN BLOOD PARAMETERS IN RAINBOW TROUT (*Oncorhynchus mykiss* Walbaum, 1792) NATURALLY INFECTED WITH *Lactococcus garvieae*

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Abstract: In a commercial rainbow trout farm located in the Aegean region, an outbreak characterized with haemorrhages in the eyes and fins, uni/bi lateral exophthalmia and darkening of skin was observed. Ten samples each were taken from clinically symptomatic fish and from healthy fish kept in a seperate ponds and these fish were grouped and marked as Diseased (D1-10) and Control (C1-10), this was followed by bacteriological examinations. Pathogenic bacteria was not isolated from asymptomatic fish but *Lactococcus garvieae* was isolated and identified with conventional and molecular methods from all clinically symptomatic fish samples. Along with bacteriological examinations, blood samples of both groups were analyzed for certain parameters with a automated blood count device calibrated for fish blood. As a result of the analyses; values for White Blood Cell (WBC), Red Blood Cell (RBC), Haemoglobin (Hb), Platelet Total (PLT), Mean Platelet Volume (MPV) and Platelet Distribution Width (PDW) in the diseased group were found to be lower than the control group ($p<0.01$, $p<0.001$)

Keywords: Blood parameters, *Lactococcus garvieae*, Rainbow trout *(Oncorhynchus mykiss* Walbaum, 1792)

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Öz:

Lactoccus garvieae İle Doğal Enfekte Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) Bazı Kan Parametrelerinin Araştırılması

Ege bölgesinde ticari gökkuşağı alabalığı yetiştiriciliği yapılan bir işletmede, gözlerde kanama ve uni-bilateral ekzoftalmus, yüzgeçlerde haemoroji ve deride kararma ile karakterize bir hastalık tablosu gözlendi. Klinik olarak hastalık belirtisi gösteren on balık ve farklı bir havuzda yetiştirilen ve herhangi klinik semptom göstermeyen 10 balıktan kan örneği alındı ve bu balıklar Hasta (D 1-10) ve Kontrol (C 1-10) grubu olarak işaretlenerek bakteriyolojik muayeneye alındı. Klinik semptom göstermeyen balıklardan patojen izole edilmezken, klinik semptom gösteren balıkların tamamından *Lactococcus garvieae* izole edilerek konvansiyonel ve moleküler yöntemlerle identifiye edildi. Bakteriyolojik muayenelerle birlikte her iki gruba ait kan örnekleri balık kanına kalibre edilmiş otomatize kan sayım cihazı kullanılarak bazı kan parametreleri yönünden araştırıldı. Sonuç olarak enfekte bireylerdeki Beyaz Kan Hücreleri (WBC), Kırmızı Kan Hücreleri (RBC), Hemoglobin (Hb), Total Trombosit (PLT), Ortalama Trombosit Hacmi (MPV) ve Trombosit Dağılım Aralığı (PDW) kontrol gruba göre düşük bulundu ($p<0.01$, $p<0.001$).

Anahtar Kelimeler: Gökkuşağı alabalığı *(Oncorhynchus mykiss* Walbaum, 1792), Kan parametreleri, *Lactococcus garvieae.*

Introduction

L. garvieae from Lactococcus genus of Streptococcaceae, is Gram positive non-motile lactic acid bacteria which produce α- haemolytic colonies in blood agar. They are non spore-forming, non-acid fast cocci which are catalase and oxidase positive (Ravelo *et al.* 2003; Vendrell *et al.* 2006). Lactococcosis caused by *L. garvieae* is an infectious disease of cultured rainbow trout and outbreaks of this disease generally occur during summer months when water temperatures rise above 16°C (Facklam and Eliot, 1995). Non specific symptoms of haemorrhaging and congestion are seen in *L. garvieae* infections (Kusuda *et al*. 1991; Domenech *et al.* 1996) and the others symptoms are immobility, darkeninig of skin and exophtalmia (Collins *et al*. 1984).

Conventional microbiological methods are often used in *L. garvieae* identification (Austin and Austin, 2007; Koneman *et al*. 1997; Timur and Timur, 2003) but identification with these methods are inefficient and time consuming (Holt *et al*. 1994). Polymerase Chain Reaction (PCR) is reported to be an easier and faster identification method for this agent (Zlotkin *et al*. 1998).

Blood parameters of fish are affected by many factors, among which water quality and infectious diseases rank first. They show significant changes in many septicemic bacterial and viral infections such as Motile Aeromonas Septicemia (MAS), Flavobacteriosis, Vibriosis, Infectious Haematopoietic Necrosis (IHN), Infectious Pancreatic Necrosis (IPN) and Viral Hemorrhagic Septicemia (VHS). These changes in blood parameters is due to losses such as haemorrhagia and the impact of the infectious disease on vital organs such as kidney, spleen, liver and pancreas (Austin and Austin, 2007; Vosyliene, 1996).

The aim of this work is to investigate the impact of Lactococcosis that naturally ocured in rainbow trout on certain blood parameters.

Materials and Methods

Sampling

The outbreak was seen in rainbow trout weighing 200-300 g. in a farm located in the Aegean region of Turkey in June, 2012. Ten diseased (D1-10) and 10 clinically asymptomatic fish (C1-10) were used in this work. For blood sampling, the fish were anesthetized with 2-phenoxyethanol (Sigma) at a concentration of 0.30 ml L^{-1} and blood from tail fins of diseased and control fish were was drawn asceptically into containers with EDTA.

Isolation and identification of bacteria

Liver, spleen and kidney samples of fish were inoculated on Trypticase-soy agar (TSA, LABM), Blood Agar (LABM) and incubations in 25°C for 48 hours were carried out. After incubation, colonies were purified and identified ac-

cording to their physiological, biochemical and enzymatic characteristics (Holt *et al*. 1994).

Genotypic confirmation of isolates

Isolates were confirmed by PCR (Zlotkin *et al*. 1998). DNA Extraction was carried out with boiling method (Çiftçi *et al*. 2009) According to this method colonies grown in TSA were suspended in DPEC-treated water (DNase-RNase free) and and was boiled for 10 min. in 100 °C. This was followed by a centrifuge step in 10.000 rpm for 10 minutes. Supernatan was discarded and remains in the tube were used as the template DNA. In PCR amplification pLG-1 (5'-CATAACAATGAGAATCGC-3') ve pLG-2 (5'-GCACCCTCGCGGGTTG-3') oligonucleotide primers were used. DEPC-treated water, 1XPCR Buffer, 1.5 Mm $MgCl_2$, 0.2 Mm of each d NTP, 1.0 U Taq polymerase, 1 µM of each primer and 5 µl template DNA was used in the PCR mastermix. The amplification consisted of 35 cycles and the steps were an initial denaturation in 94°C, followed by a 1 min. denaturation in 94°C, 1 min of annealing in 55°C, an extension of 1.5 min. in 72°C and 1.5 min.of final extension in 72°C. As a result, 1100 bp long amplification product was considered to be positive. Amplification products were visualized on a 1.5% agarose gel and 100 bp. DNA marker was used. Positive and negative controls were *Lactococcus garvieae* ATCC 43921 and *Enterecoccus faecalis* ATCC 29212, respectively.

Investigation of blood parameters

After aenesthesia, blood from tail fins of diseased and control fish were was drawn asceptically into containers with EDTA. Blood samples were analyzed for with a blood count device calibrated for fish blood (Mindray BC 2800, Turkey). White Blood Cell, (WBC), Red Blood Cell, (RBC), Haemoglobin (Hb), Platelet Total Value, (PLT), Mean Platelet Volume, (MPV) and Platelet Distribution Width (PDW) values were researched.

Statistical analyses of blood parameters

For the statistical analyses of data; SPSS (for Windows Release 11.5 Standart Versiyon Copyright © Spss Inc. 1989-2001) was used. With an independant sampling test, data on blood parameters in these two groups of fish were compared. The $P<0.05$ were accepted significant.

Results and Discussion

Clinical symptoms for Lactoccosis in fish were haemorrhages in different parts of the body, darkening of skin and exophtalmia in some (Figure 1). In necropsy, anemia in liver and splenomegaly was observed. Gram stained slides prepared from internal organs of infected fish revealed Gram positive bacterial colonization (Figure 2) whereas similiar findings were not observed in control group fish.

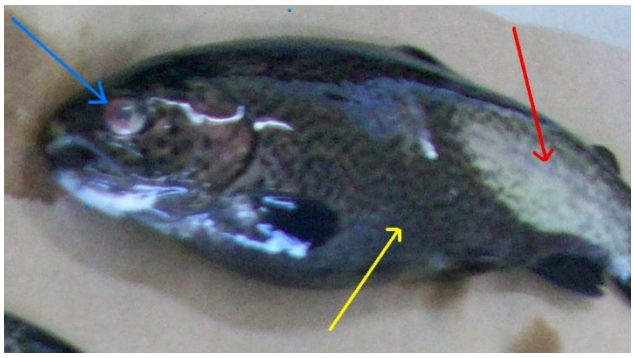

Figure 1. Rainbow trout infected with *L. garvieae*. Red Arrow; ulceration on body, yellow arrow; darkening of skin, blue arrow; exophtalmia

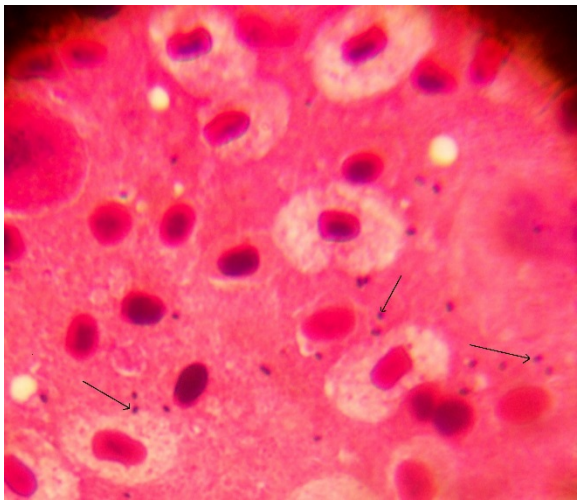

Figure 2. Gram positive cocci in a Gram stained slide prepared from the spleen of an infected fish.

Although *L. garviae* was isolated from all diseased fish, no bacterial pathogens were detected in asymptomatic fish. No differences were observed in phenotypical characterization of isolates and results are summarized (Table 1). Afterwards, PCR confirmation was carried out (Figure 3).

Table 1. Phenotypical characterization of *L. garvieae* isolates

Colony size	<1mm	Growth in % 0 NaCI	+
Bacterial size (Average)	0.4-0.7 µm	Growth in % 6 NaCI	+
Motility	-	Growth in % 8 NaCI	+
Oxidase	-	Lysine Utilization	_
Catalase	-	Ornithin Utilization	_
Pigment production	-	Arginin Utilization	+
Haemolysis in sheep blood	A	Starch	_
Simmon's Citrate Agar	-	Gelatin liquefication	_
Nitrate production	-	Aesculin hydrolysis	+
Methyl red	+	Lactose	_
Voges-Preskauer	+	Glucose	+
Indole	-	Fructose	+
H_2S prodcution	-	Galactose	+
Growth in 4°C'	+	Mannitol	+
Growth in 30 °C'	+	Sucrose	+
Growth in 37 °C'	+	Sorbitol	_
Growth in 45 °C'	+	Rhamnose	_
Growth in Mac-Conkey agar	+	Inositol	_

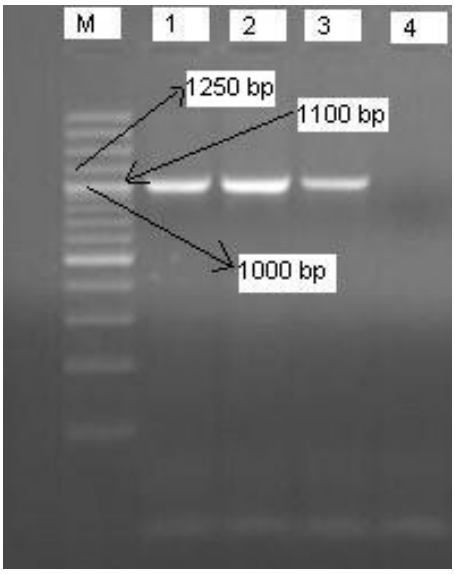

Figure 3. *Lactococcus garvieae* specific PCR, 1100 bp. M; 100-bp DNA ladder, 1: *Lactococcus garvieae* ATCC 43921, 2-3: isolates. 4: *Enterecoccus faecalis* ATCC 29212

Statistical analyses of WBC, RBC, HGB, PLT, MPV ve PDW values obtained from *L. arvieae* infected and control fish (Table 2).

Veterinary haematology is more common in small animal practice; research on blood parameters in fish is limited (Dethlof *et al*. 1999) and analyses are generally carried out manually (Vosyliene, 1996; Cakıcı and Aydın, 2006; Handy *et al*. 1999; Zorriehzahra *et al*. 2010). As fish erythrocytes have nuclei, standard blood count devices record them as leukocytes. Thus, for fast and reliable results with automated devices; the system has to be calibrated with standard (fish) blood. Besides, blood sampling in fish requires special skills as veins are invisible and hard to manipulate. As a result of all these hindrances; determining blood parameters of fish haematology is harder than other animals.

Table 2. Statistical analyses of certain blood parameters of *L. garvieae* infected and control group fish.

Parameters	Control ($X \pm S_{\bar{x}}$) n=10	Infected ($X \pm S_{\bar{x}}$) n=10	P
WBC ($10^3/mm^3$)	29.36 ±0.22	21.88 ±1.12	***
RBC ($10^6/mm^3$)	0.57 ±0.05	0.11 ±0.01	***
HGB (g dL^{-1})	11.30 ±050	5.65 ±0.35	***
PLT (10^9 L^{-1})	75.12 ±4.39	44.77 ±5.11	***
MPV(fL)	5.24 ±0.23	4.36 ±0.06	**
PDW(fL)	17.85 ±0.44	16.30 ±0.06	**

(*P<0.05, **P<0.01, ***P<0.001)

Investigating haematological parameters is an effective way in determining the health status of fish (Blaxhall, 1972; Rehulka, 2002; Martins *et al*. 2008). Erythrocyte (RBC) count in fish naturally infected with *L. garvieae* was found to be significantly lower than the control group. Similar findings of lower RBC values were also reported in coho salmon (*Oncorhynchus kisutch*) infected with *V. anguillarum*, in rainbow trout infected with *Aeromonas sobria, A. caviae, Aeromonas/Streptococcus, Y. ruckeri* and *V. anguillarum*, in tilapia infected with *Streptoccus iniae*, in carp infected with *A. hydrophila* and also in Asian chyclid fish (*Etroplus suratensis*) with epizootic ulcerative syndrome (Harbell *et al*. 1979; Barham *et al*. 1980; Altun and Diler, 1996; Pathiratne and Rajapakshe, 1998; Rehulka, 2002; Harikrishnan *et al*. 2003; Chen *et al*. 2004; Ceylan and Altun, 2010). In Nile tilapia experimentally infected with *Enterococcus sp*., RBC values were reported to be unchanged (Martins *et al*. 2008).

Rainbow trout infected with lactococcosis were also found to have lower WBC values (p<0.001). Although in early stages of experimentally induced Yersiniosis and Vibriosis, leukocyte counts were reported to rise, they were seen to decline as the disease progressed (Altun and Diler, 1996; Ceylan and Altun, 2010). However, Martins *et al*. (2008), have observed increasing WBC values in Nile tilapia experimentally infected with *Enterococcus sp.*

Haemoglobin counts were reported to be significantly lower in rainbow trout infected with *Y. ruckeri, Vibrio anguillarum, A. salmonicida, Aeromonas/Streptococcus*, in Atlantic salmon with cold water Vibriosis, in coho salmon (*Oncorhynchus kisutch*) with *V. anguillarum* and in Asian Chyclid fish (*Etroplus suratensis*) with epizootic ulcerative syndrome (Harbell *et al*. 1979; Barham *et al*. 1980; Altun and Diler, 1999; Pathiratne and Rajapakshe, 1998; Ceylan and Altun, 2010). HGB values obtained in this work were seen to be lower in rainbow trout due to lactococcosis (p<0.001).

Different researchers have noted important increase in platelet counts in experimentally infected with *Y. ruckeri, V. anguillarum, Aeromonas salmonicida* and *Renibacterium salmoninarum* and in Nile tilapia infected with *Enterococcus sp* (Bruno and Munro, 1986; Demirdöğen, 1997; Altun and Diler, 1999; Ceylan and Altun, 2010; Martins *et al*. 2008).. In this research, Lactococcosis infection in rainbow trout was found to lead to a decrease in platelet count

Conclusion

Values for White Blood Cell, (WBC), Red Blood Cell, (RBC), Haemoglobin (Hb), Platelet Total Value, (PLT), Mean Platelet Volume, (MPV) and Platelet Distribution Width (PDW) in rainbow trout naturally infected with *Lactococcus garviae* were found to decrease by Control group (p<0.01, p<0.001).

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SHORT COMMUNICATION KISA MAKALE

LARVAL ORGANOGENESIS OF *Schizothorax zarudnyi* (Nikolskii, 1897) (Cyprinidae): HISTOLOGICAL ASPECTS

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Abstract: The present study describes histological aspects of the development of gills, heart, kidney, swim bladder and spleen from Day 0 up to Day 21 after hatching in Hamun mahi *Schizothorax zarudnyi* larvae and provides valuable information on its structural status during ontogeny. This information is particularly useful for establishing the functional systemic capabilities and physiological requirements of larvae for optimal welfare and growth. Observations described are related to main developmental stages of Hamun mahi and are defined on the basis of external morphological features. Pronephric excretory structures, a tubular heart and respiratory anlage are present at hatching but spleen and swim bladder become apparent during Stage 2. During the first two stages, Hamun mahi larvae undergo intense organogenesis particularly during Stage 2. Despite Stage 2, the next developmental stage is not characterized by the appearance of new structures but by the increase in size and complexity of the pre-existing ones.

Keywords: Ontogenesis, Gill, Heart, Kidney, Swim bladder, Spleen

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Öz: ***Schizothorax zarudnyi*** **(Nikolskii, 1897) (Cyprinidae)'nin Larval Organogenezisi: Histolojik Özellikler**

Bu çalışma, hamun mahi (*Schizothorax zarudnyi*) larvalarında yumurtadan çıktıktan sonra 0. günden 21. güne kadar geçen sürede solungaç, kalp, böbrek, yüzme kesesi ve dalak gelişiminin histolojik özelliklerini tanımlamaktadır ve türün ontogenez sırasındaki yapısal durumu hakkında değerli bilgiler sağlamaktadır. Bu bilgiler özellikle larvaların yapısal sistemik kabiliyetlerinin ve en uygun refah koşulları ile gelişimi için gerekli fizyolojik ihtiyaçlarının belirlenmesinde fayda sağlamaktadır. Bahsedilen bulgular hamun mahi balığının temel gelişim basamakları ile ilişkilidir ve dış morfolojik özelliklerine göre tespit edilmiştir. Pronefrik salgı yapıları, tübüler bir kalp ve solunum sistemi taslağı yumurtadan çıkışta bulunmaktadır fakat dalak ile yüzme kesesi 2. safhada belirgin hale gelmektedir. Hamun mahi larvaları ilk iki safha boyunca, özellikle de 2. safhada yoğun bir organogenezis geçirmektedir. 2. safha, daha ileri bir gelişimsel safha olmasına rağmen yeni yapıların görülmesiyle karakterize edilmemekle birlikte, daha önceden var olan organların boyca büyümesi ve daha karmaşık bir hal almasıyla tanımlanmaktadır.

Anahtar Kelimeler: Ontogenez, Solungaç, Kalp, Böbrek, Yüzme kesesi, Dalak

Introduction

The rapid increase in per capita fish consumption in low-income, populous countries has made identification and utilization of new methods of aquaculture necessary to fulfill this demand. *S. zarudnyi*, is a fast growing endemic cyprinid which is found in the Sistan region in Iran (Abdoli, 2009). Hamun mahi feeds on aquatic plants (~75%), some animal foods (~25%) and detritus. Despite the ecological and commercial importance of Schizothoracine, there are only a few studies on them (Yongxing, 1997; Mir & Channa, 2009; Mir & Channa, 2011), and Kalbassi et al. (2008) extensively analyzed the karyotype in *S. zarudnyi*.

Some hatchery programs were developed by The Iranian fisheries over a decade ago with the goal of restocking and stock enhancement after the severe drought of Hamun Lake in 2000. However, high mortality rate throughout larval rearing in intensive culture conditions, particularly during the period of exogenous feeding, has up to now hampered the process of Hamun mahi culture.

The health and quality of spawners and environmental conditions during egg incubation determine the amount and quality of the endogenous reserves of larvae, and the environmental and feeding conditions affect the rate of larval development (Hachero-Cruzado et al., 2009). Rearing conditions, such as temperature and optimal feeding protocols, are critical for obtaining adequate survival and growth rates as well as optimal larval organogenesis and metamorphosis (Gisbert et al., 2008).

Structural and functional changes in tissue, organs and body system are established during larval development. Therefore, invention of new methods of aquaculture is heavily dependent on the knowledge of larval development. The larval ontogenesis can be divided into different stages according to the main developmental events (Blaxter, 1988).

Although the basic mechanisms of organ development are similar in all teleosts, there are inter-specific variations in the timing of organ formation, development and functionality (Hachero-Cruzado et al., 2009). Therefore, it becomes necessary to carry out specific organogenesis studies for different species to optimize larval-rearing techniques and feeding conditions for fish (Gisbert et al., 2008; Zambonino Infante et al., 2008).

The aim of this study is to describe the ontogenetic development of some organs including respiratory system, kidney, heart, spleen and swim bladder in *Schizothorax zarudnyi* larvae from hatching to Day 21 for a better understanding of their organization and functionality with a view to provide a basis for future aquaculture studies of Hamun mahi as an alternative culture species.

Materials and Methods

Larval rearing conditions and sampling

Schizothorax zarudnyi larvae were obtained by natural spawning from a broodstock adapted to captivity at the Hatchery and Aquaculture

Center of Zahak in Sistan & Baluchestan, Iran. The larvae reared from hatching to 21 DAH (days after hatching), at a water temperature of 17-18.5℃, in 100 -liter tanks at density of 50 individuals per liter in an open circulation under a natural photoperiod. The larvae were fed from 5 DAH to 10 DAH with a mixture of milk powder and chicken egg yolk, and from Day 11 onward with a mixture of artemia nauplii and BioMar® fish meal. The larvae were randomly sampled and fixed in Bouin's solution for subsequent histological studies.

Light microscopy

The samples were fixed in Bouin's fixative for 24 hours, then were washed and dehydrated in an ascending series of ethanol for embedding in paraffin. Following embedment in paraffin, transversal and longitudinal sections of 4 µm were cut on a SLEE Mainz CUT 6062 microtome, collected on glass slides, and stained with a mixture of eosin-haematoxylin, fushcin and methylene blue.

Results and Discussion

Hamun mahi Growth

Growth in length was assessed by measuring Absolute Growth Rate (AGR) as $mm.day^{-1}$ and Specific Growth Rate (SGR) as $\%.day^{-1}$ (Hopkins, 1992). Equations used are as follows:

$$AGR = (L_f - L_i)/t \quad (1)$$

and

$$SGR = [(Ln_{Lf} - Ln_{Li})/t] \times 100 \quad (2)$$

where L_f is the mean length of the sample in mm at the end of each developmental stage, L_i is the mean length at the end of the previous stage, and t is the duration of the stage in days. The highest AGR and SGR values were recorded during Stages 1 and 2, and the lowest during Stage 3, in conjunction with the endogenous reserves exhaustion at the end of Stage 2 (Table 1). A schematic synthesis of the main ontogenetic events occurring in *S. zarudnyi* related to each developmental stage is shown in Table 2. Table 3 shows changes in TL of Hamun mahi larvae during their first 21 days of life. During this period, fish growth was not uniform. Newly hatched larvae were measured 8.57±0.81mm reaching 11.79±0.58mm by the end of the period studied.

Main ontogenetic stages during larval development

According to the source of food and the external morphological features, three main stages were established during *Schizothorax zarudnyi* larval development (Table 1). First Stage or exclusively lecitotrophic (endotrophic) period (0-2 DAH), second stage or lecitoexotrophic (endoexotrophic) period (3-8 DAH) and third stage or exclusively exotrophic period (9-21 DAH).

During the first two stages, the larvae underwent intense organogenesis which was particularly prominent in Stage 2. Despite the first two development stages, the main characteristic of Stage 3 was the increase in size and complexity of pre-existing organs and not the appearance of new structures.

Gills and Pseudobranch

Gills anlages were visible in the pharyngeal region at hatching (Figure 1a). Four pairs of primordial gill arches, formed by cores of chondroblast and covered by an undifferentiated epithelium, were evident at the beginning of Stage 2 (Figure 1b). First vascular structures exhibiting blood cells inside were seen by 3 DAH (Figure 1c). Primordial lamellae were first observed in filaments carried by the second and third gill arches appeared by 5 DAH (Figure 1d). The first mucous cells were observed in the gill filaments epithelium at 5 DAH (Figure 1e). The filaments were evident from 3 DAH and increased in length afterwards. Cartilaginous axis and taste buds appeared later by 10 DAH (Figure 1e, f).

During Stage 2, pillar cells delimited lamellar vascular structures and chloride cells gathered at the base of the lamellae and inside the interlamellar spaces of the filament. The pseudobranch was first seen as paired structures located in the anterior zone of the branchial cavity at the beginning of Stage 2 (Figure 1b). During Stage 3, both the filaments and the lamellae increased in length and number. The pseudobranch continued to develop during Stage 3 with its cartilaginous skeleton appearing at 9 DAH. During Stage 3, the gill differentiation was also completed.

Heart

The heart was already seen at hatching as a tubular structure located at the anterior part of the coelomic cavity, just below the gill anlage. Also blood cells were seen within the chambers (Figure 2a).

At the beginning of Stage 2, the valve between the atrium and the ventricle developed and the four compartments became visible in the heart: atrium, ventricle, bulbus arteriosus, and sinus venosus (Figure 2b). The atrium oriented towards the ventricle. Latter it presented a thin wall and a big lumen with blood cells inside. From 3 DAH, first trabeculae were seen in the ventricle (Figure 2b). During Stage 2 (from 7 DAH), the atrioventricular valve was completely formed in conjunction with the proliferation of the ventricular trabeculae (Figure 2c).

The atrial trabeculae were visible from early Stage 3 or 12 DAH (Figure 2d). The valves between sinus venosus-atrium and ventricle-bulbus arteriosus were first observed at Stage 2 and achieved its complete formation by the end of the last stage.

Table 1. Morphological aspects of the larval ontogeny of *S. zarudnyi*

Stage	Age (DAH)	SL (mm) (mean±SD)	AGR (mm day^{1-})	SGR (%day^{1-})	Food source	Morphological and structural observation
1. Lecitotrophic	0-2	8.57 ±0. 81	0.716	8.375	Endogenous	Yolk sac; Undifferentiated digestive tract; Mouth closed.
2. Lecitoexotrophic	3-8	10.62 ±0. 71	0.315	2.982	Endo-exogenous	Differentiated digestive tract; Opening of the mouth.
3. Exotrophic	9-21	11.79 ±0. 58	0.015	0.133	exogenous	Yolk reserves exhausted.

Table 2. Schematic synthesis of the main ontogenetic events occurring in *Schizothorax zarudnyi* larva during endotrophic (Stage 1), endo-exotrophic (Stage 2) and exotrophic (Stage 3) periods. Numbers in brackets show number of days after hatching.

Stage	Endogenous reserves	Digestive tract	Accessory glands
1 [0-2]	Homogenous acidophilic YS surrounded by VE; Fragmentation of yolk started [2].	Mouth closed; straight tube not differentiated into regions; No MC present.	Present at hatching as a mass of undifferentiated cells; gallbladder anlage visible.
2[3-8]	YS granular; Complete resorption [8].	Mouth opened; gut regionalization [3]. PH developing; MC & TB in oesophagus & intestine; enterocytes with microvilli [4]; Valve formation [5]; Vacuolization of gut evtrocytes; gut differentiated; MC & TB proliferation.	Bile canaliculi & gallbladder evident; Acinar organization of the pancreas & accumulation of ZG; Sinusoids with blood cell inside; Vacuolisation of HC.
3 [9-1]	Exhausted; remnant of VE evident till 10 DAH.	Developed PH; Intestinal folds evident [10].	Increase in size of both organs; Increased vacuolisation of HC & proliferation of sinusoids.

Number in Brackets indicate the age of the larva in days after hatching (DAH). BC, blood cells; HC, hepatocytes; MC, mucous cells; PH, pharyngeal teeth; TB, taste buds; VE, vetellins envelope; YS, yolksac; ZG, zymogen granules.

Table 3. Growth of *Schizothorax zarudnyi* larvae during 21 days. (DAH, days after hatch; TL, total length; SL, standard length).

DAH	SL	TL
1	7.86	8.78
2	9.29	10.18
3	9.66	9.93
4	10.26	10.76
5	10.24	10.97
6	10.87	11.71
7	11.16	11.90
8	11.55	12.26
9	11.55	12.51
10	11.67	12.26
11	11.33	12.52
12	11.29	12.14
13	11.38	12.49
14	11.37	12.60
15	11.18	12.23
16	12.20	13.51
17	12.29	13.42
18	12.57	13.69
19	12.54	13.60
20	12.24	13.29
21	11.75	12.60

Kidney

The kidney, which was already present at hatching, consisted of several primordial pronephric tubules running just below the notochord axis and a few hematopoietic cells between them (Figure 3a).

During Stage 2, primary renal tubules appeared. Also their convolution is started. Increasing in hematopoietic tissue was seen in this stage (Figures 3b, c). The renal tubules were covered internally by an epithelium composed of cubical cells with a large, centrally or basally located nucleus and cilia or microvilli on the apical surface (brush border). Each renal corpuscle was formed by a glomerulus and Bowman's capsules at 8 DAH (Figure 3d).

The structure of the pronephros at Stage 3 was similar to that of Stage 2 except for a noticeable increase in hematopoietic tissue. At the end of Stage 3, two regions were identified in the kidney: the anterior portion, occupied mainly by hematopoietic tissue; and the caudal region where developing mesonephric elements were dominant.

Swim bladder

At the beginning of Stage 2 (3 DAH), primordial swim bladder differentiated from the dorsal wall of the digestive tract (Figure 4a). Gas glands were also become apparent from 3 DAH. The rete mirabile became more evident from 4 DAH (Figure 4a, b). Swim bladder epithelial lining was formed from columnar cells similar to those of the gut and its structure began to inflate by 5 DAH (Figure 4c). At 7 DAH, the swim bladder lumen was observed to be connected to the digestive tube through the pneumatic duct (Figure 4d). The swim bladder was more structured during Stage 3.

Spleen

Spleen anlagen were first seen during the lecitoexotrophic period at 5 DAH (Stage 2). They initially consisted of loose small spherical clusters of mesenchymal cells located at the vicinity of the liver (Figures 5a, b). At the same stage, the organ started to acquire its elliptic shape, as development proceed, the spleen being formed at this time by mesenchymal and hematopoietic basophilic cells and surrounded by pancreatic tissues. Moreover, the splenic ellipsoids and sinusoids were visible during Stage 3 (Figures 5c, d).

The histological study presented in this paper provided information on the internal structure, and therefore, the functional status of larval *S. zarudnyi* during its different ontogenetic stages observed at ''arbitrarily chosen moments" (Osse & van den Boogaart, 1999) of an essentially continuous process of development distinguished by the appearance or disappearance of discernible histological characters.

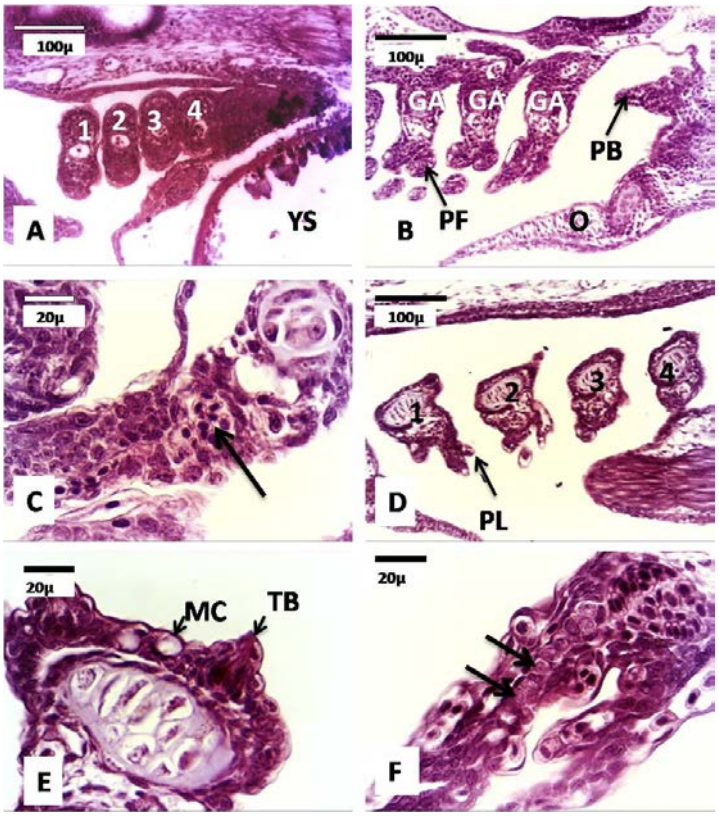

Figure 1. Microsections of the branchial cavity of *Schizothorax zarudnyi* larvae during development. (A) Gill primordial (1-4) at the beginning of stage 1 ; (B) gill arches, primordial filaments and pseudobranch at early stage 2; (C) vascular structures with blood cells (3 DAH); (D) Primordial lamellae at 5 DAH; (E) taste buds and mocus cells on the gill arch at 10 DAH; (F) cartilaginous axis of filament was seen at 10 DAH. MC, mocus cell; GA, gill arch; O, operculum; PB, pseudobranch; PF, primordial filament; PL, primordial lamellae; TB, taste bud; YS, yolk sac.

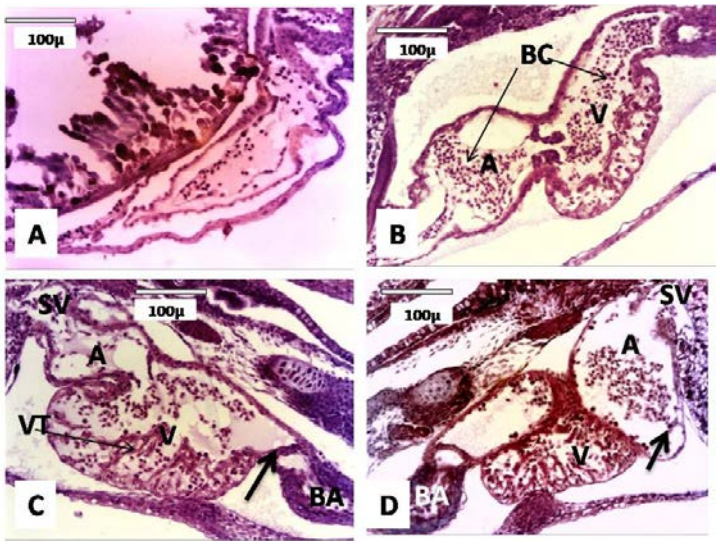

Figure 2. Microsections of the heart of *Schizothorax zarudnyi* larvae during development. (A) primordial heart at the beginning of stage 1; (B) compartmentilization of heart at the beginning of stage 2; (C) proliferation of the ventricular trabeculae , the valve between ventricle and bulbus arteriosus is visible in the picture (7 DAH); (D) Apearance of the first atrial trabeculae at 12 DAH. A, atrium; BA, bulbus artriosus; BC, blood cell; SV, sinus venosus; V, ventricle; VT, ventricular trabeculae.

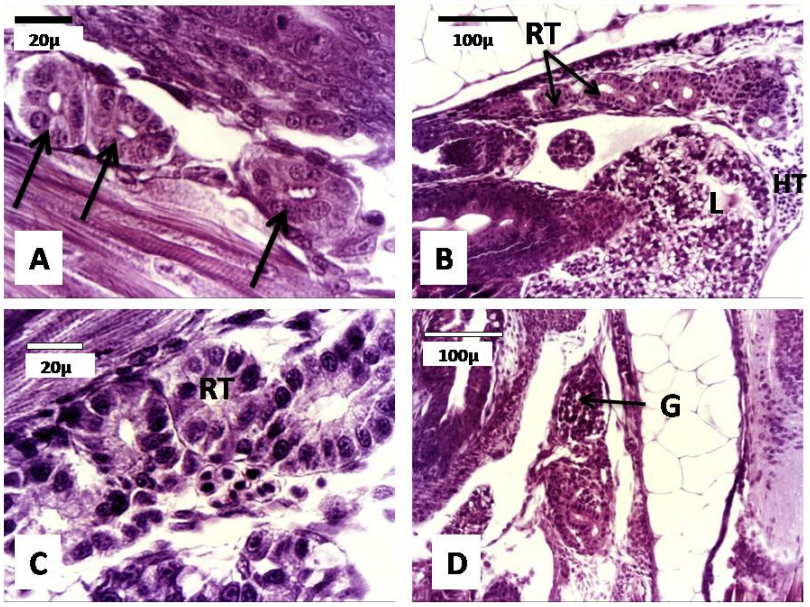

Figure 3. Microsections of the kidney of *Schizothorax zarudnyi* larvae during development. (A) primordial pronephric tubules at the beginning of stage 1; (B,C) primary renal tubules at the beginning of stage 2, (D) renal corpuscle with glomerulus and bowmans capsule are apparent from 8 DAH. G, glomerulus; HT, haematopoietic tissue; L, liver; RT, renal tubule.

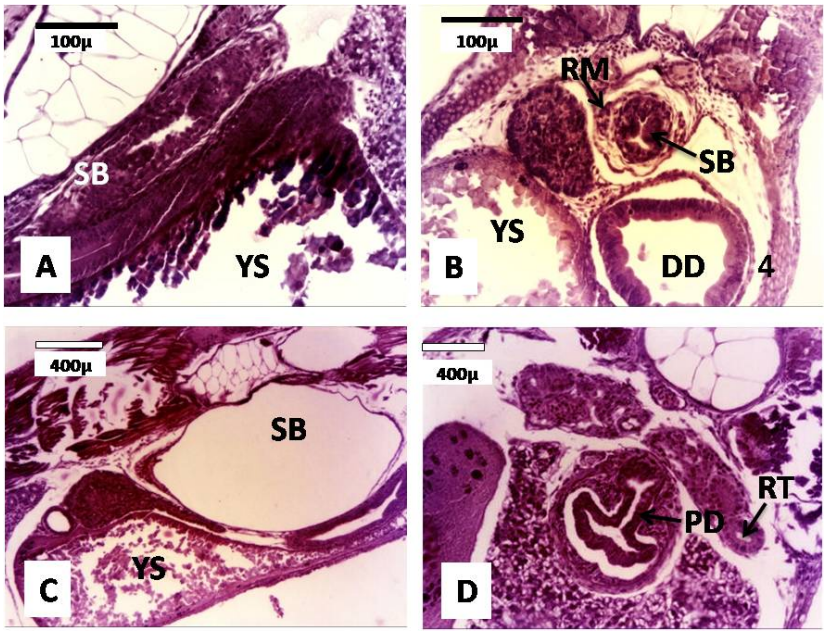

Figure 4. Microsections of the swim bladder of *Schizothorax zarudnyi* larvae during development. (A) Defferentiation of swim bladder at the beginning of stage 2; (B) Apearance of rete mirable at 4 DAH; (C) inflation of swim bladder at 5 DAH; (D) pneumatic duct is seen at 7 DAH . DD, digestive duct; PD, peunomatic duct; RM, rete mirable; RT, renal tubule; SB, swim bladder; YS, yolk sac.

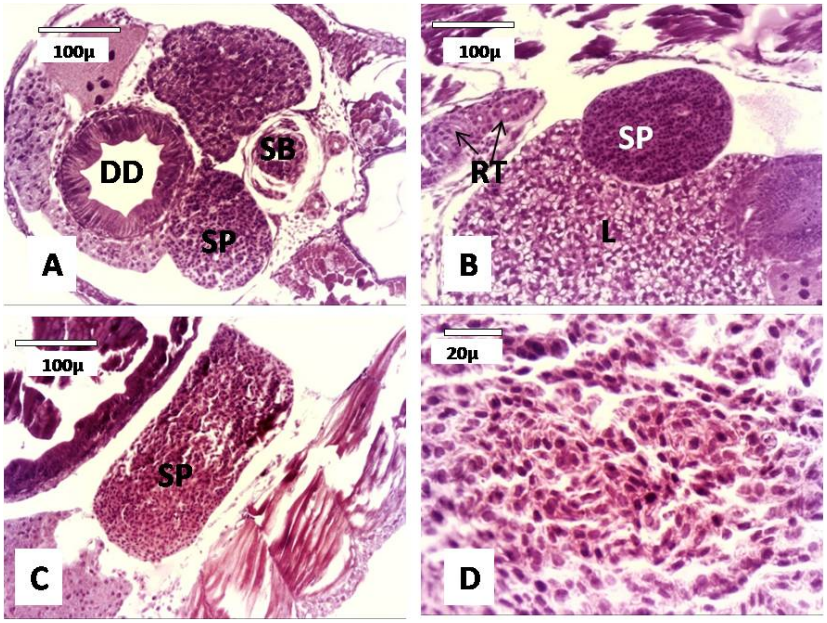

Figure 5. Microsections of the spleen of *Schizothorax zarudnyi* larvae during development. (A, B) spleen anlage at lecitoexotrophic period (5DAH); (C, D) structured spleen with ellipsoids and sinusoids at 11 DAH. DD, digestive duct; L, liver; RT, renal tubule; SB, swim bladder; SP, spleen.

From the tabular overview presented here, it is apparent that Hamun mahi larvae undergo intense organogenesis during the first two stages particularly during early Stage 2. On the contrary, Stage 3 is not characterized by the appearance of new structural elements but by quantitative changes experienced by pre-existing structures. Although the basic mechanisms of larval development do not differ greatly among teleosts, there is some interspecific variability in the timing at which the different ontogenetic events occur (Blaxter, 1988; Hachero-Cruzado et al., 2009).

In this study, during Stage 1, primordial kidney, heart and gills were present (at 0 DAH) and developed dramatically. This shows the importance of such organs to larval development in early days of life.

Osmoregulation is assured in larvae as in any other fish species (Walles & Tytler, 1996) by chloride cells located on the whole body surface of the larvae particularly in the buccopharyngeal epithelium (Santamaria et al., 2004). Also it is recognized that the gill becomes functional when lamellae develop. Comparing with most teleosts which develop lamellae only in the second half of exotrophic stage, in Schizothrox larvae, lamellae appear during Stage 2 (Lecitoexotrophic period) at 5 DAH (Santamaria, 2004; Sanchez-Amaya et al., 2007; Hachero-Cruzado et al., 2009). This shows a fast-developed gill system so that there is no intense morphological event in the respiratory system during Stage 3 except for increase in length and number of filaments and lamellae.

At 3 DAH, the circulatory and excretory system became functional in Schizothorax larvae mainly due to the compartmentalization of the heart. The swim bladder started to inflate on Day 5 after hatching similar to what was reported by Unul et al. (2001). In contrast, in sole (Boulhic and Gabaudan 1992) and coregonid larvae (Loewe and Eckmann, 1988), this inflation happens several days later. The inflation time of the swim bladder may be important in catching of living prey (Unul et al., 2001).

Spleen is one of the most important lymphoid organs of teleosts. In freshwater teleosts spleen is the last organ to become lymphoid. However, in marine teleosts the order in which major lymphoid organs develop is kidney, spleen and finally, the thymus (Zapata, 2006). Our observations on spleen development of Hamun mahi is similar to that of freshwater fish (Walles and Tytler, 1996; Zapata, 2006).

Conclusion

In summary, the study of developmental steps of Hamun mahi (*Schizothrox zarudnyi*), reveals that the larvae follows a similar ontogenic pattern as in other teleost fish species, although some interspecific differences in the timing of organ and system development were observed.

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We dedicate this work to deceased Co-author, Hossein Abbasi. Also Authors would like to thank Elham Karimi for technical assistance at SBU and Dr. Mostafa Nayyerloo at GNS Science, New Zealand for his invaluable editorial comments on the paper and proofreading.

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

A RAPID HPLC METHOD FOR DETERMINATION OF 4-HEXYLRESORCINOL RESIDUES IN SHRIMP

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Abstract: A rapid method for the extraction, separation and quantification of the 4-hexylresorcinol from shrimp with high performance liquid chromatography (HPLC) is described. This modified method is faster than previously described methods with short pre–preparation and sharp resolution in HPLC procedures (sample preparation, extraction, separation and quantification total 40 min.). The coefficients of determinations (r^2) measured for 4-hexylresorcinol were obtained over 0,999 in each curve. The confidence interval of the recovery working range of 1.5–2.5 mg/kg was approximately 95%. Limits of detection and quantitation were determined as 0.04 and 0.06 mg/kg for shrimp.

Keywords: 4-hexylresorcinol, Shrimp, Melanosis, Residue levels, HPLC

Öz: **Karideslerde 4-hexylresorcinol kalıntılarının belirlenmesi için hızlı bir HPLC Metodu**

Bu makalede, karidesten 4-hexylresorcinol'un ekstraksiyonu, yüksek basınçlı sıvı kromotografisinde (HPLC) ayırımı ve miktarının belirlenmesi ile ilgili hızlı bir metot anlatılmıştır. Bu modifiye metot, kısa ön hazırlık ve HPLC' de keskin pik ayırımı ile daha önceki metotlara gore daha hızlıdır (örnek hazırlığı, ekstraksiyon, ayırım ve miktarın belirlenmesi toplamda 40 dakika). İki eğride de korelasyon katsayısı (r^2) 0,999 un üzerinde ölçülmüştür. 1.5–2.5 mg/kg lık geri kazanım çalışma aralığının güvenilirlik değeri yaklaşık % 95 bulunmuştur. Tespit limiti ve ölçüm limiti 0.04 ve 0.06 mg/kg olarak bulunmuştur.

Anahtar Kelimeler: 4-hexylresorcinol, karides, Melonosis, kalıntı seviyesi, HPLC

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Introduction

Color change called "melanosis (black spots)" consists in shrimp and crustacean as a result of enzyme activity during storage (Figure 1.). Melanosis has no health risk for consumer. This color change is the main reason for economic loss in world shrimp trade which is accepted as spoiled product in the eyes of the consumer. For the solution of this trouble, many chemicals have been developed for commercial applications. One of them is 4-hexylresorcinol that its use has increased in recent years. 4-hexylresorcinol prevents from polyphenol oxidase (PO) that it is found under shrimp shell and in it. Thus, melanosis is delayed. But 4-hxylresorcinol remains residue such as other chemicals and its usage is limited with legal limit values (Collins-Williams 1983; McEvily et al. 1991; Montero et al. 2006, Mendes et al. 2006). Maximum acceptable residue levels for this agent in raw material in China and Canada is 1 mg/kg, whereas it is accepted as 2 mg/kg in European Union (GB 2760-1996, 1996; Food and Drug Regulation 1078, 1998; EU Scientific Committee on Food, 2003).

Figure 1. Melanosis formation in shrimp (Varlık et al., 2007)

4-hexylresorcinol is a new additive in fisheries because it inhibits development of enzymatic browning melanosis in a variety of storage situations which developes rapidly in shrimp, in lobsters after catching and during iced storage (Montero et al. 2004; Mendes et al. 2006).

The detection and quantification of 4-hexylresorcinol in shrimp are achieved by HPLC. Reverse phase HPLC method coupled with florescence detector is preferred for quantification of 4-hexylresorcinol levels in food, because of its better selectivity, accuracy and sensitivity.

The objective of this study was to investigate determination of 4-hexylresorcinol levels in shrimp flesh compared to other methods in terms of analysis time and sensitivity.

Materials and Methods

Samples

Deep water pink shrimp samples (*Parapenaeus longirostris*, Lucas 1846) were collected from the Marmara Sea, Turkey.

Determination of 4-hexylresorcinol Residues

4-hexylresorcinol value was analyzed by a HPLC method modified from Jonker and Dekker (2000).

Chemicals and laboratory equipments: 4-Hexylresorcinol (%99) (Acros Organics, Catalog No. 197920250, Belgium), Methanol (Merck gradient grade for liquid chromatography LiChrosolv® Reag. Catalog No. 1.06007.2500, Germany), Acetonitrile (Merck gradient grade for liquid chromatography LiChrosolv® Reag. Catalog No. 1.00030.2500, Germany), Potassium dihydrogen phosphate (Merck Catalog No. 1.04873.1000, Germany), Ortho – Phosphoric Acid (Merck Catalog No. 1.00573.1000, Germany), Deionized water, 0.22 µm, 13mm syringe membran type nylon filters (E-Chrom Tech, Taiwan), Volumetric flask (25 mL and 50 mL), Measuring cup (10 mL, 25 mL and 100 mL), Automatic pipette (10 µL, 100 µL and 1000 µL), Syringe (10 mL) and Centrifuge tube (50 mL)

Instrument: Shimadzu LC 10 AT Vp series pump, Shimadzu SIL 10AD Vp cooling automatic sampling (4°C), Shimadzu RF 10AXL fluorescence detector (FLD), Shimadzu CTO 10AV Vp, Shimadzu SCL 10A Vp and Shimadzu Class-Vp 6.14 (Shimadzu Corporation, Japan).

Chromatographic conditions:

Injection volume: 50 µL

Flow rate: 0.8 mL/min

Mobile phase: 40/60 (Phosphate buffer solution (0.01 M Phosphate buffer solution was prepared by dissolving 1.36g KH_2PO_4 in 1 L deionized water and was adjusted to pH 3 with %25 H_3PO_4)/Acetonitrile)

Detector: Fluorescence (Excitation wavelength: 280nm, Emission wavelength: 310nm)

Column: ACE C_{18} 250mm x 4.6 mm x 5µm (Advanced Chromatography Technologies Ltd, Scotland)

Column oven temperature: 35°C

Analysis time: 10 min

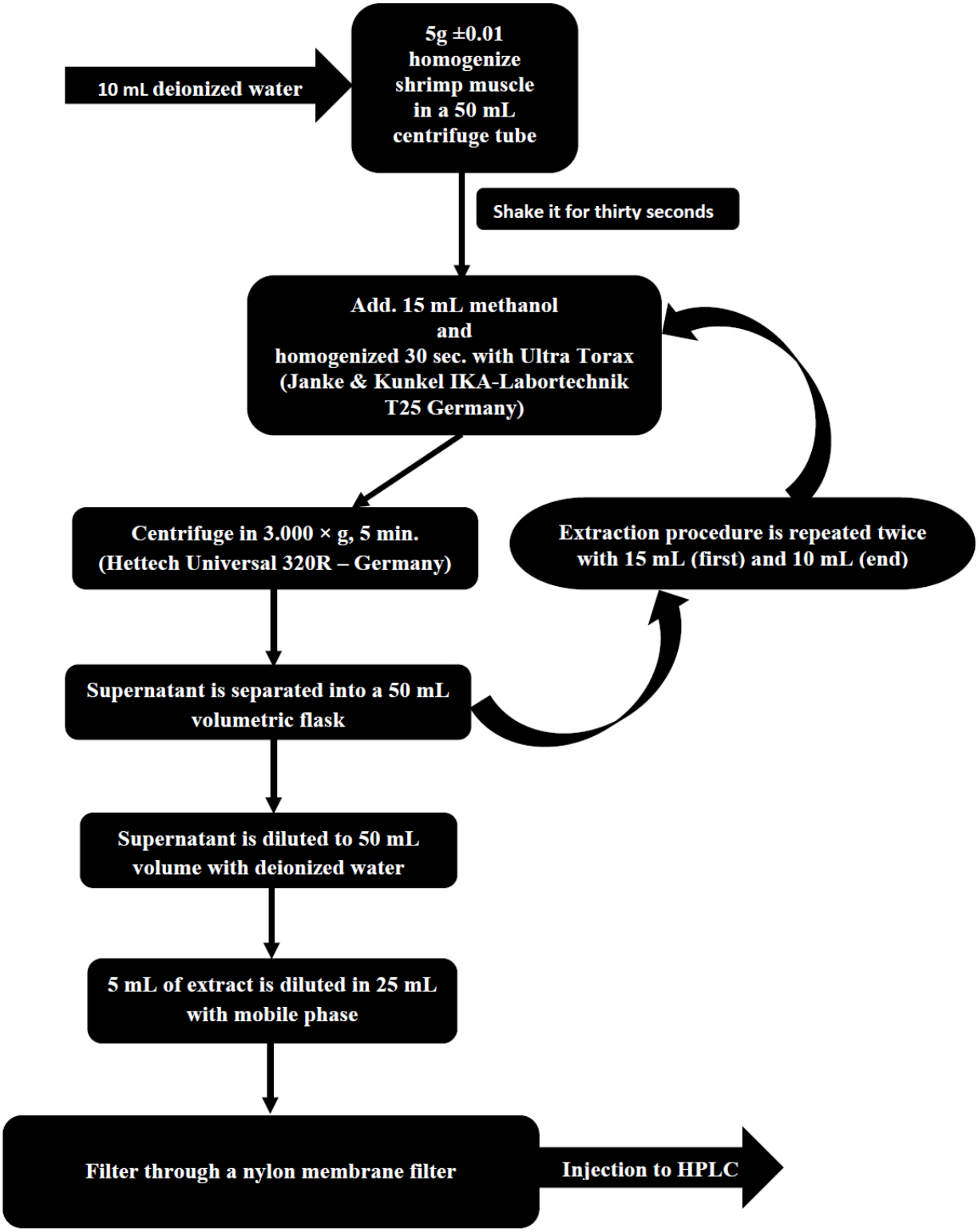
Standard solution: Stock standard solutions (500 ppm) of 4-hexylresorcinol was prepared by dissolving 50 mg in 100 mL of methanol. The standard solution can be stored for at least 6 months in brown glass (in freezer -18°C). Buffer stock solution (5 ppm) was prepared by dissolving 1 mL stock standard solution in 99 mL of methanol. Working standard solutions in the concentration of 1, 5, 10, 50, 100, 250, 500 and 1000 ppb were prepared daily by dilution of buffer stock solution in mobile phase.

Calculation: The concentration of 4-hexylresorcinol (µg 4-hexylresorcinol/L) was calculated from the regression equation derived from the standard curve.

4-hexylresorcinol was calculated from the equation:

$$4-\text{hexylresorcinol (mg/kg)} = \frac{HPLC\ value\ read\ (\mu g/L) \times 0.250\ L}{Sample\ weight\ (g)}$$

Analysis steps:



Method Performance Criteria

Limit of detection (LOD), Limit of quantification (LOQ), accuracy (recovery- trueness, repeatability and within reproducibility – precision) have been studied to determine method performance criteria by single laboratory validation.

LOD and LOQ: LOD and LOQ were determined by analyzing 10 independent fortified samples at a very low level and calculated with mean plus 3 standard deviation and 10 standard deviation, respectively.

Accuracy: For accuracy study, two levels spiked samples were prepared and 18 replicates have been performed in each level. 1 mL of 4-hexylresorcinol was added into blank samples from 7,5 mg/L and 12 mg/L standard solutions to obtain 1.5 and 2.5 mg/kg concentrations respectively. Samples have been stored in a dark and ambient temperatures for 2 h before extraction. For each level, 18 samples were analyzed as 6 replicates in three independent analytical runs. Repeatability and within reproducibility were calculated according to ISO 5725-2 (1994) expressed as coefficient of variation. Recovery was calculated according to Eurachem Guide and expressed as percentage.

Linearity: Two calibration curves were prepared at 5 concentration levels and triplicate measurements at each level (**Curve 1**: 1, 5, 10, 50 and 100 ppb, **Curve 2**: 100, 250, 500, 750 and 1000 ppb) by linear regration. 4-hexylresorcinol concentration in samples were determined with the aid of the instrument software by using the calibration curve.

Validation scheme: The present method was optimized and validated according to "The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics" (Eurochem Guide-1998).

Statistical Analysis

Results were presented with mean and standard deviation of values (n=3) in the tables. The standard deviation and coefficient of determinations (r^2) were determined using STATISTICA 7 (StatSoft Tulsa, Oklahoma-USA).

Results and Discussion

4-hexylresorcinol in shrimp

Typical HPLC chromatograms of 4-hexylresorcinol were presented in Figure 3 and Figure 4. 4-hexylresorcinol was separated at 280 nm (excitation) and 310 nm (emission) wavelength in less than 10 min.

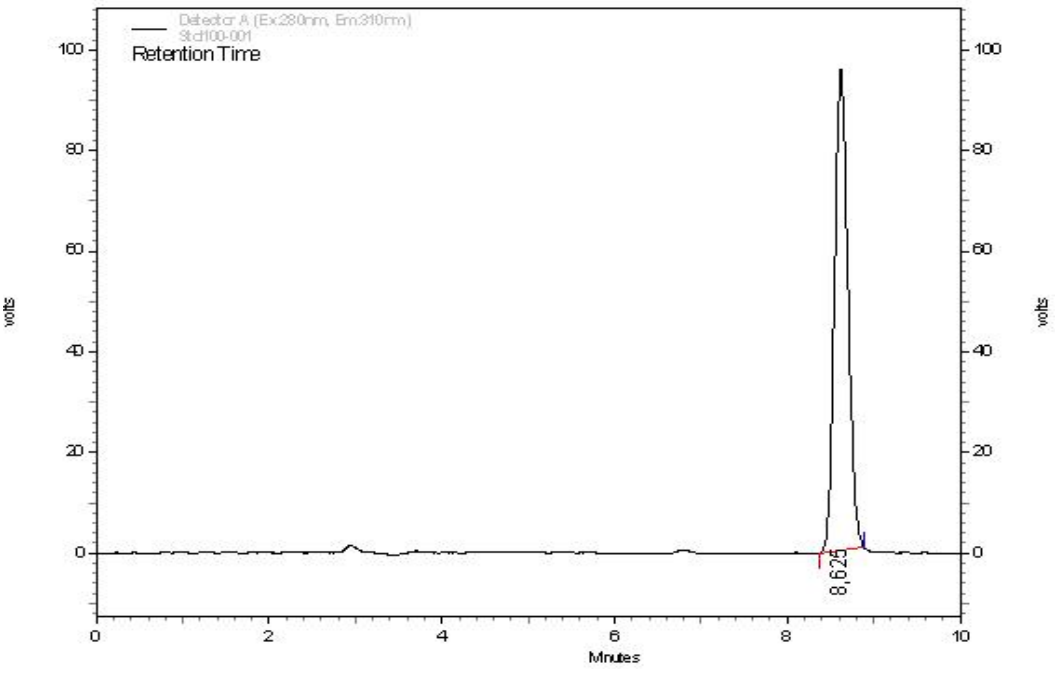


Figure 2. 4-hexylresorcinol standards peak

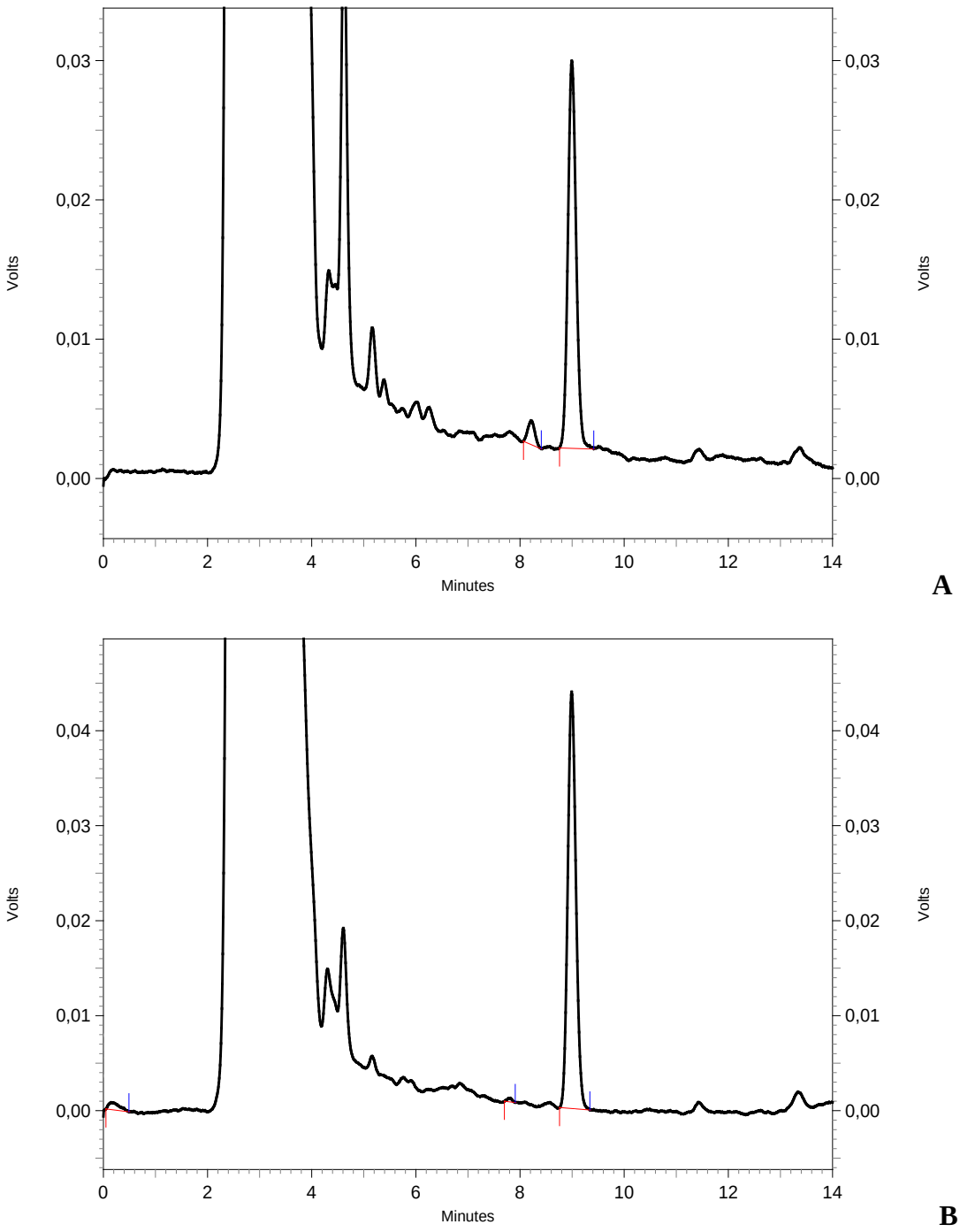


Figure 3. 4-hexylresorcinol spiked shrimps chromatograms at the levels of 1.5 (A) and 2.5 (B) mg/kg

LOD and LOQ

LOD and LOQ values of the method for 4-hexylresorcinol were presented in Table 1.

Table 1. LOD and LOQ of the method for 4-hexylresorcinol

LOD (mg/kg)	LOQ (mg/kg)
0.04	0.06

Accuracy

The recovery, repeatability and reproducibility ranged between 94.68 to 94.80%, 1.23 to 2.51% (CVr), and 1.89 to 2.74% (CVR), respectively (Table 2). General acceptance range is between %70-110 for recovery studies and recovery obtained in this study was good in trueness. These results show that the method has a good precision due to its high recovery and low CV_r and CV_R values.

Table 2. Results for recovery, repeatability (CV_r) and reproducibility (CV_R) in 4-hexylresorcinol

	N	1.5 (mg/kg)	2.5 (mg/kg)
Day1	6	1.41	2.37
Day2	6	1.45	2.41
Day3	6	1.42	2.34
Mean		1.43	2.37
SD (±)		0.04	0.04
CV_r (%)		2.51	1.23
CV_R (%)		2.74	1.89
Recovery (%)		94.68	94.80

Linearity

Regression analysis was performed for calibration and correlation coefficients (r^2) were obtained over 0,999 in each curve (Table 3).

Jonker and Dekker (2000) have found the pooled recovery 81.6%. The confidence interval was ranged from 81.6 % to 95 %. The relative standard deviation (RSD) for the range of application (1.5–2.5 mg/kg) was 2.1 %.

Table 3. Linearity of 4-hexylresorcinol

4-hexylresorcinol	Regression equation	r^2
Linearity 1	$9.69477x10^5$	0.999795
Linearity 2	$9.72366x10^5$	0.999106

With this work, we decreased the time for preliminary preparations and increased sensitivity of the study.

Conclusions

It can be concluded that the presented method (HPLC-Fluorescence) has potential to be used for quantification of 4-hexylresorcinol in shrimps due to its rapidness, simplicity, reliability and sensitivity. The validated method has a good overall recovery, repeatability and reproducibility and also low LOD and LOQ value. It can separate 4-hexylresorcinol at 280 nm (excitation) and 310 nm (emission) wavelength in less than 10 min and also provides minimal sample preparation. The average analysis time (sample preparation, extraction, separation and quantification) takes approximately 40 min.

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FULL PAPER TAM MAKALE

EFFECTS OF STOCKING WITH BROOD FISH TO MANAGE RESIDENT STREAM DWELLING BROWN TROUT *Salmo* cf. *trutta* L. STOCK

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Abstract: Management with the indigenous stock of resident brown trout *Salmo* cf. *trutta* of the Danubian strain in the protected area of the River Gradac with the catch-and-release fishing regime since 2000 resulted in the prime fly fishing stream in Serbia. Stocking with the brown trout parr in 2007 in the density issued by fisheries management plan supplemented with the large-sized brood fish beyond the issued fisheries measures in the upper section of the River Gradac was followed by change in structure of fish community and population structure of brown trout there. The time of maturation of brown trout was delayed and their size at maturation increased in 2008, due to the abrupt decrease in the density of their stock. That change did not affect an increase of brown trout mean weight, but only an increase of their average age, with the decrease in relative abundance, relative biomass and annual natural production, implying the prolonged recovery period of brown trout stock in the upper section of the River Gradac. In contrast to that, stocking with brown trout parr only in the density issued by fisheries management plan was beneficial on the second, downstream situated section. Another prominent feature that followed stocking as a management measure was the introgression of brown trout of the Atlantic lineage into the gene pool of brown trout of the native, Danubian lineage, as confirmed in wild-bred brown trout.

Keywords: Brown trout, Stocking brood-fish, Effects on Population Structure, Gene Pool Introgression

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Öz:

Damızlık Kahverengi Alabalık (*Salmo cf. trutta*, L.) Stoğunun Bölgesel Akarsu Stok Yönetimine etkileri

Sırbistan'da, Tuna nehrinin kollarından biri olan Gradac nehrinde yerel kahverengi alabalığın (*Salmo cf. trutta*) bölgesel stoğunun kontrol altına alınması sayesinde bu bölge sportif avcılığın merkezi haline gelmiştir. Bu nehrin üst bölümlerinde 2007 yılında gerçekleştirilen balıklandırma çalışmaları beklenen daha fazla büyük boy anaç bireyin gelişmesine olanak sağlamıştır ve bunu takiben bu bölgedeki balık populasyonlarının yapısında değişiklikler ortaya çıkmıştır. 2008 yılında kahverengi alabalıkların ergenleşme süreleri uzamış ve anaç bireylerin boylarında artış görülmüştür. Bu değişiklikler kahverengi alabalıkların ağırlık ortalamasını değiştirmemiş fakat yaş ortalamalarında değişikliklere neden olarak bu bölgedeki stokların düzelmesinde gerekli sürenin artmasına neden olmuştur. Bunun aksine, yönetim planındaki kahverengi alabalık yavrularının izin verilen miktarı aynı nehrin alt bölgesinde başarıya ulaşmıştır. Stoklamanın sonucunda ulaşılan verilerden biri de bölgede bulunan Danubian soyuna Atlantik soyunun karışmış olmasıdır.

Anahtar Kelimeler: Kahverengi Alabalık, Anaç Stoklaması, Populasyon Yapısı Üzerine Etkiler, Gen Aktarımı

Introduction

It was common in the fishery management that human activities posed a strong risk of certain kinds of adverse effects on native stocks, e.g., compromising the original biodiversity and loss of intraspecific variability after an introduction of non-native strains of brown trout and change in genetic composition of their wild stock, i.e., the decrease of the indigenous genetic variability from interbreeding (White, 1989; Ryman *et al*., 1995), or disturbance of life-history features that might affect also the fisheries value of native brown trout stock. For the management implications it is important to notice that in many species fitness is affected by physical performance, which eventually also links to morphology (Huckins, 1997; Belk *et al*., 2007). Fish stocking is to be considered an important fishery management, as well as conservational activity that is to be accomplished as a way of compensating the shortfalls in recruitment, or in threat of extinction by target species that arises through overfishing in the Catch-and-Remove fishing regime applied, or through environmental degradation. Where fish are stocked into self-reproducing populations of the same species, the effects of stocking is difficult to evaluate, since the understanding the population dynamics is particularly challenging when the populations are supported by a mixture of stocking and natural reproduction. Compensatory responses in growth and mortality are likely to reduce the absolute contribution to recruitment from natural stocks and the contribution to reproduction from stocked fish is often uncertain, especially when the stocked fish are from the strain not adapted to the recipient water (Welcomme, 2001). When they belong to the strain that shows invasive character, they quickly compromise the indigenous character of native stocks by introgression, as revealed frequently in both worldwide (Laikre and Ryman, 1996, Laikre *et al.*, 1999) and local fishery management with brown trout *Salmo trutta* (L., 1758) stocks (Marić *et al.*, 2006; Razpet *et al.*, 2007). Both for economic and population dynamics issues, the juveniles of hatchery fish have been almost exclusively used for stocking. However, their hatchery produced morphology, probably insufficient for a natural stream environment, the most likely result was poor survival in the wild (Vehanen *et al*., 2009). Several kinds of impact by stocked brown trout on the native ones were recognized also in the UK (Anonymous, 2003), e.g., competition and predation by stocked fish, stimulation of influx of predators, stimulation of fishing effort and thus an excessive exploitation of wild stocks, as well as the introduction of diseases and change in genetic composition of wild stocks due to interbreeding. The more recently promoted Catch-and-Release fishing regime fundamental to sports in recreational fishery in temperate countries enabled easier model of fishery management, although it can make certain damages from hook setting and releasing that might result in proneness to diseases and feeding difficulties, which might lead to the increased mortality rate. This regime showed especially positive effects in conservation of indigenous stocks. For that reason, it is widely applied in the management with wild trout stocks.

Considering the significance of brown trout stocks in fishery and conservational senses, it is

desirable to assess characteristics of brown trout from the fish caught and released unharmed back into its habitat. Sticking to that often makes the investigation of certain characteristics (e.g., reproductive characters) causing damage, or sacrificing of fish, very difficult. Since the knowledge of those characteristics is usually necessary for the evaluation of the status of brown trout stocks, it was important to find a way to investigate them to minimize the harmful effects of data collection on brown trout. In accordance with the theory of saltatory ontogeny (Balon, 1975; 1990), shifts from one to the next developmental period have been shown to be followed by more-or-less abrupt alteration of various characteristics (e.g., habitat use and morphology) in various fish species, e.g. in ruffe *Gymnocephalus* spp. (Kováč 1994), stone loach *Barbatula barbatula* L. (Kováč *et al*., 1999) and minnow *Phoxinus phoxinus* (L.) (Simonović *et al*., 1999). Following that, Simonović *et al*. (2000) demonstrated that abrupt alteration in modes of growth (both in length and in weight) in huchen *Hucho hucho* (L.), detected by Piecewise Linear Regression (Nickerson *et al*., 1989), at both smaller (i.e. late juvenile period) and trophy sizes (i.e. late adult period), corresponds well to the ages of first maturation, i.e. the onset of adulthood (detected breakpoints at 3.005 kg and 49.6 cm *Sl*) and of the onset of senescence (detected breakpoints at 16.541 kg and at 110.0 cm *Sl*), respectively. In addition to that, Simonović and Nikolić (2007) revealed that size at which brown trout attain maturity coincides very well with the breakpoint values obtained for resident brown trout from two streams in Serbia, as learnt from the records obtained in two examinations accomplished for the prosecuting of poachers. The similarity of Fulton Coefficient (F_c) values for both smaller and larger huchen from the catching reports at the River Drina fishing area in the 2005 – 2007 time period to those from the 1998 – 1999 time period and in allometry occurring for growth –in-length and -in-weight for smaller and larger huchen, respectively (Simonović *et al*., 2011), strongly validated the use of Piecewise Linear Regression, i.e., the breakpoints of size-related variables for detection of developmentally-related life-history events in fisheries research. Simonović and Nikolić (2007) reported that resident brown trout in the River Gradac matured at very large size in comparison to the stocks from the smaller streams. They stated that age structure of the population would rise, i.e., the maturation would be attained at greater body size if the river depth was greater at constant abundance and biomass of brown trout, or reversely, the maturation would be attained at smaller body size if abundance would rise at constant biomass of brown trout, i.e., the age structure of the population would change by drop of average age. So, greater the mean individual length, i.e., the lower the relative density, the maturation would be attained at greater size. Jenkins *et al*. (1999) reported the strong negative relationship between the individual size and density of brown trout, suggesting that large trout are competitively advantageous in comparison to smaller ones when density increases.

The first aim of this study was to test the null hypothesis (H_0) that stocking of brown trout of the brood size did not affect the population structure and life-history features (mean weight, average age, relative abundance, relative biomass and annual natural production, time of maturation) of native brown trout in the River Gradac. Considering the strictly applied Catch-and-Release fishing regime during the whole six-years management period and lack of records about (i.e., negligible rate of) illegal fishing, as well as the approximately same CPUE and structure of brown trout catch, it seems that fisheries pressure and rate of poaching can be excluded as factors that affected the change in population structure in brown trout from the River Gradac, and that stockings, especially that one accomplished with the brood size fish in the distinct, upstream section of the stream, were the events that impacted the change in the population structure of brown trout there.

In the beginning of the management period in 2001, there was no information about the molecular status of brown trout in the River Gradac, yet. Before the stocking events in 2007 (in 2005, when the sampling for the molecular analyses was worked out by fly fishing), it was learnt that brown trout of Atlantic (At) lineage, of the Atcs1 haplotype already occur in the River Gradac in approximately one-third of the population (which should be taken with caution, due to the small number of samples processed) (Marić *et al*. 2006). Considering this, the more recent molecular status of brown trout in the River Gradac was also wanted to be ascertained for, in order to check how much brown trout of the At lineage already introgressed into the gene pool there. Hence, in the 2008 sampling, fin clips form only two brown trout were taken, since the majority of brown trout sampled then looked peculiar (Figure

1, A) in compare to both commonly colored fish of the presumably Danubian lineage (Figure 1, B) and those of the strange and strong punctuation featuring brown trout of the Atlantic lineage (Figure 1, C). Considering that no legal justification for this action was seen at the moment of sampling, it was not possible to take fin clips from more fish.

Figure 2. One (of two) brown trout sampled at the locality D in 2008 for RFLP screening (A) of about 30 cm in *Sl*, typical brown trout of Danubian lineage of about 25 cm in *Sl* caught by fly fishing on 4 May 2007(B) and typical brown trout of Atlantic lineage of about 40 cm in *Sl* caught by fly fishing on 9 June 2007(B) (the length of the cork handle of the rod is about 20 cm), both close to the locality M at the upper section of the River Gradac.

Materials and Methods

Study Site and Fisheries Management Plan

River Gradac is a capacious freestone stream situated in the vicinity (about 100 km to south-west) of Belgrade, the capital of Serbia (Western Balkans, south-eastern Europe) (Figure 2). It is about 14 km and of approximately same size all along, due to extensive water capturing from majority of numerous springs that used to feed it. It flows through the picturesque gorge and the whole landscape was proclaimed a natural protected area at early 1990s. The upper section is considered the part from the spring to the Monastery Ćelije and downstream section down to the dam at the area of Degurić village is considered the middle section. The size (width, depth and water discharge) of the River Gradac in those two sections is about the same, despite of the nearly 5 km of distance between them, due to the extensive water capturing from the springs for the local water-supplying systems. Occurrence of two dams (one of them being the concrete one, of the height over 10 m) in that section lacking any fish pass facilities makes the downstream migration between brown trout stock much more (if not the only) likely than in the reverse direction. In addition to the scope of brown trout stock fishery management and supplemental to it, the conservational scope on the brown trout stock is also important, considering that River Gradac Gorge is the nature protected area obliged by national legislation and specific management documents to maintain indigenous character of animal and plant species.

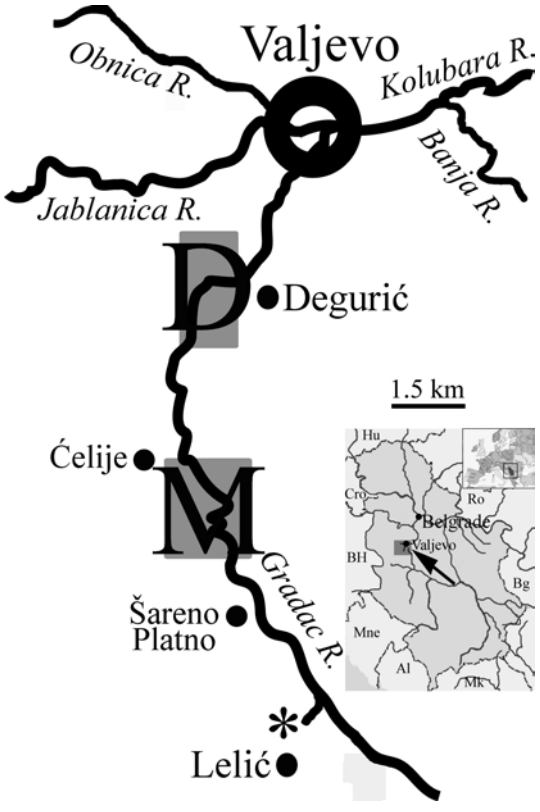

Figure 2. Situation of sampling localities M and D on the River Gradac (asterisk denotes the spring of the River Gradac, upstream of which is the river bed that has a water flow in wet seasons only) and location of the area of investiga-

tion in the region (Serbia) and in Europe.

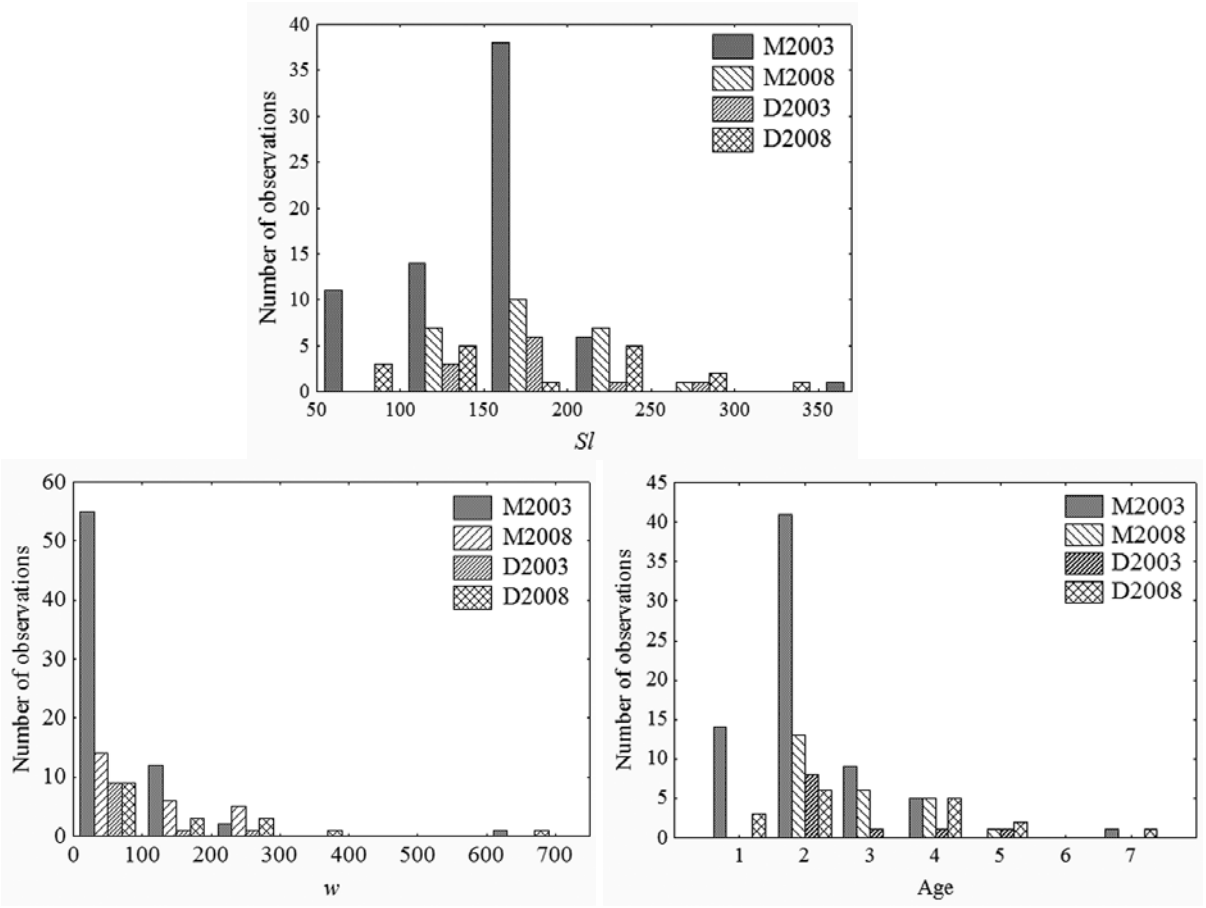

Figure 3. Frequencies of particular standard length (*Sl*), weight (*w*) and age classes of brown trout in each of samples from the River Gradac.

The last Fisheries Management Plan (FMP) for the River Gradac (Simonović *et al*., 2003) set the main issues for the fishery management in the next five-year time period. It strictly imposed a necessity for the preservation of indigenous brown trout of Danubian (Da) mtDNA lineage (after Bernatchez *et al*., 1992; Bernatchez, 2001) in the nature protected area where it is situated. Unfortunately, in the time of the preparation of that FMP, it was not known that native brown trout stock in the River Gradac was already compromised by stocking with the 1+ hatchery reared brown trout of Atlantic lineage in 2001, as revealed by Marić *et al*.(2006), who detected afterwards that one out of three brown trout sampled from the River Gradac was of the Atcs1 haplotype. The FMP also issued the strict "Catch-and-Release" fly fishing of then extremely abundant brown trout without any stocking as a precautionary, conservation-related measure that was to minimize a risk of introduction of domesticated, hatchery-reared brown trout strains (e.g., of the Atlantic lineage) that are alien for this area, already used in the closely situated Slovenia for fish stocking for a long time (Marić *et al*., 2011).. The possibility of stocking in the FMP was restricted only to an occasion of strong spring torrents washing out the newly hatched brown trout parr. That fishing regime without additional stocking was enforced until 2007. In the late spring 2007, after the strong public pressure from the fly-fishing community on the fishery manager and without the real need (i.e., without any high spring water level and deleterious effect, but only for public reasons), about 1.8 x 10^4 hatchery-reared brown trout yearlings, i.e., parr of up to 10 cm in standard length (*Sl*) were introduced in two stocking occasions along the upper and middle sections of the River Gradac. However, on the second stocking occasion, 10^2 brown trout of the average weight (*w*) of about 1 kg, which were the brood fish from the hatchery, were stocked exclusively in the upper section of the River Gradac. Stocking with the brood-size brown trout

was worked out beyond the directives given in the FMP and legally issued procedure for stocking. There was no checking of genetic status of either yearlings, or brood fish that were stocked then, since there was no legally issued obligation for that.

Sampling Methods

Records from the two samplings of brown trout at the River Gradac were compared. The first sampling was accomplished on the preparation of the FMP, in 18 and 19 August 2003, using the engine-powered (2.4KW) electrofishing gear Suzuki-Bosch™ (220V DC, *I* = 6A max.), and the second was in 16 and 17 July 2008, using the battery-powered portable electrofishing gear AquaTech™ IG200/1® (380 / 600 V DC, *I* = 15A max.). On both samplings, the same two sampling sites were worked out: one in the upper section, in the area upstream of the Monastery Ćelije (here denoted M), and the other in the middle section, in the area of the Degurić village (here denoted D). At each sampling site, three stream sections that were of the habitat structure (riffle and pool), length (up to 150 m) and time of working (not exceeding 30 minutes), i.e., of the fishing effort as similar as possible, were electrofished for two upstream passes, with stop-nets spread both at starting and ending fishing points. Since brown trout are considered important in both fishery and conservational sense, as well as there was no need either from the point of legislation, or research to take fish samples, they were returned alive to the same stream section they were sampled from.

Data Collecting

Brown trout samples landed during electrofishing were stored at large mobile plastic container both held in and filled with the stream water. At the end of sampling at each section, each fish was measured for its standard length (*Sl*) using the measuring tape to the nearest millimeter and weight (*w*) using the digital scale Philips (of accuracy 1 g). Scales for ageing were taken from the left flank above the lateral line and fish were released alive to the stream. Scales were examined using the Carl Zeiss binocular magnifier under the 25 times of magnification.

Statistical Analyses

Data on catch-per-unit-of-effort (CPUE) for brown trout and all other fish species from both sampling sites were used to calculate their relative abundance (in ind ha^{-1} unit), whereas relative biomass and relative annual natural production (in kg ha^{-1} unit) were calculated for brown trout only, after Ricker (1975), from average values obtained at each sampling site. Mean age of samples ($\bar{a}$) was calculated according to the age structure (i.e., frequency of occurrence of each age) of each sample, following the expression:

$$\bar{a} = \sum \frac{a_i n_i}{N},$$

where a_i is the age of fish, n_i is the frequency of occurrence of that age in the sample and *N* is the total number of fish of all ages in each sample. Difference in age structure between trout samples from two localities and years of sampling was tested using χ^2 test. The speed of growth, both –in *Sl* and -in *w* in brown trout samples at two localities in each sampling year was assessed using the linear regression on log-transformed *Sl* and *w*. Pairwise tests between samples from different years for their means and regression coefficients were accomplished using *t*-test.

The investigation of attainment of maturity was accomplished using the Piecewise Linear Regression method of Nickerson *et al*. (1989) on log-transformed *Sl* and *w*, in order to examine whether there is an alteration in growth, i.e., the breakpoint that represents two distinct and significantly different linear regression equations: one for *y* values less than, or equal to breakpoint, and the other for *y* values greater than, or equal to breakpoint:

$$\underbrace{y = b_{01} + b_{11}x_1 + \ldots + b_{m1}x_m}_{y \le b_n}$$
$$\underbrace{+ b_{02} + b_{12}x_1 + \ldots + b_{m2}x_m}_{y \ge b_n}.$$

Molecular Analysis

The molecular (i.e., lineage) status after Bernatchez (2001) of brown trout was ascertained by the method of Restriction Fragment Length Polymorphism (RFLP) of Berg and Farris (1984) from the anal fin clips which were taken from two fishes sampled at the locality D and stored in the 96% ethanol. That small number of samples was due to lack of legal obligation to assess genetic status of brown trout and strict limitation to sample fin clips for an analysis that came from the fisheries inspector who surveyed the sampling. A total DNA sample was obtained using the High Salt Extraction Technique of Miller *et al*. (1988). The mtDNA Control Region known also as D-loop (about 1080 bp) was amplified using primers 28RIBa (Snoj *et al*, 2000) and HN20 (Bernatchez and Danzmann, 1993). PCR was accomplished under the following conditions: initial denaturation (95°C, 5 min) followed by 30 cycles of strand denaturation (94°C, 45 s), primer annealing (52°C, 45 s) and DNA extension (72°C, 2 min; the last extension prolonged to 5 min) in the MultiGene Thermal Cycler TC9600-G-230V® (Labnet International, Inc.™). Total PCR volume of 20 µl was used, containing 2µl 10µM of each primer (Thermo Scientific™), 0.4 µl 10mM dNTPs (), 1 µl BSA, 2 µl 10 x PCR buffer (Kapa Biosystems™), 0.08 µl 5U/µl *Taq* polymerase (Kapa Biosystems™) and 1µl (about 100 ng) of genomic DNA. Amplification was checked by loading on the 2% agarose gel and running on 100V for 1 hour. Control Region was afterwards digested using the Thermo Scientific™ FastDigest® *SatI* endonuclease. Samples for the restriction reaction (10µl PCR amplified Control Region of mtDNA, 2µl 10x digestion buffer, 1 µl, i.e., 10 units of *SatI* and 17 µl autoclaved distilled water; each in total 30 µl of content) for 25 minutes on 37ºC and loaded on the 2% agarose gel with the 0.5 x TBE electrophoresis buffer, with the 25 minutes of staining with the 4µl of Applichem™ SYBR Green® each, in the dark on the room temperature. They were run for 60 minutes at 100V and visualized under the UV light (302 nm). For molecular weight standard, the Thermo Scientific ™ GeneRuler® 50bp ladder was used, together with the three controls of brown trout positively of the Da and At lineages, respectively.

Results and Discussion

The structure of fish community at the locality M appeared significantly different in the 2009 sample in relation to that from the 2003 sample (χ^2 = 1951, df = 6, p < 0.01), mainly due to an occurrence of native schneider *Alburnoides bipunctatus* and chub *Leuciscus cephalus* as new species for this river section, as well as of translocated grayling *Thymallus thymallus*, sparsely (and out of the management plan issues) stocked in 2007 at this section of the River Gradac. In addition to that, the abundance of brook barbel *Barbus balcanicus* appeared much greater than in 2003 and that of bullhead *Cottus gobio* was much smaller than in 2003. Significant change in the fish community structure at the locality D in the six year period (χ^2 = 350, df = 6, p < 0.01) was mainly due to the appearance of chub and stone loach *Barbatula barbatula* being the native fish species occurring now at it, with the much less abundance of the rest of native fish species in this river section, except the minnow *Phoxinus phoxinus*.

The 2003 sample from the site M contained 70 brown trout of range 7 cm to 36 cm in *Sl* and 4 – 640 g in *w*, whereas the 2008 sample from that site comprised 25 brown trout of ranges 12 cm to 27.5 cm in *Sl* and 26 g to 277 g in *w*. The 2003 sample from the site D contained 11 brown trout of 13 cm to 25.5 cm in *Sl* and 27 g to 237 g in *w*, whereas that taken in 2008 held 17 brown trout of 6.5 cm to 35 cm in *Sl* and 5 g to 634 g in *w* (Figure 3).

The average age of brown trout increased from 2003 to 2008 (Table 1) significantly only at the locality M (t = 2.815, df = 45, p < 0.01) and not on the locality D (t = 0.591, df = 17), but change in abundance at particular age classes (Figure 3.) resulted in significant difference between age structure of brown trout samples from 2008 and 2003, both on locality M (χ^2 = 195, df = 5, p < 0.01) and locality D (χ^2 = 150, df = 5, p < 0.01). The first prominent difference in the structure of brown trout population was that at the locality M in 2008 in compare to that in 2003 there was the absence of brown trout yearling (0+). Whereas, that age class was rather abundant at the locality D in 2008, in difference to 2003. The second obvious difference was an occurrence of brown trout of greater size (both -in length and -in weight) and older age at the locality D in 2008, in contrast to 2003 samples.

In compare to brown trout samples from 2003, those from 2008 on both localities showed the decline in relative abundance, relative biomass and annual natural production, as well as the increase in mean weight (Table 1). Decline in relative abundance was significant both at localities M (t = 4.364, df = 94, $p < 0.01$, and D (t = 3.312, df = 26, $p < 0.01$). The decline in relative biomass was significant at locality M (t = 8.894, df = 94, $p < 0.01$) but not at the locality D (t = 0.941, df = 26, $p > 0.05$). The decline in annual natural production at the locality M was significant (t = 2.375, df = 94, $p < 0.05$), and at the locality D it was not significant (t = 1.942, df = 26, $p > 0.05$). The increase in mean weight was not significant both at localities M (t = 0.303, df = 94, $p > 0.05$) and D (t = 0.578, df = 26, $p > 0.05$).

Table 1. Average age, growth in length (standard length, *Sl*) and in weight (*w*), with the growth speed (*b*) in length and in weight, relative abundance, biomass, mean weight and annual production (with standard errors) of brown trout in the River Gradac, together with the breakpoint values for the growth in length and in weight that represent the size of their maturation and growth speeds in parr, i.e., before the attainment of maturation (*b*1) and after it in adult brown trout (*b*2); *se* is standard error of mean, s_b is standard deviation of the growth speed parameter.

	M 2003	M 2008	D 2003	D 2008
Average age (years)	2.1 ± 0.12	2.8 ± 0.18	2.5 ± 0.31	3.1 ± 1.71
Age classes		*Sl* ± *se* (cm)		
0+	9.0 ± 3.53			7.0 ± 0.50
1+	16.2 ± 2.52	15.0 ± 0.43	15.0 ± 5.17	14.2 ± 0.89
2+	19.7 ± 2.06	19.8 ± 0.53	19.0 ± 0.00	
3+	22.9 ± 6.40	23.7 ± 0.20	21.5 ± 0.00	24.1 ± 0.37
4+		27.5 ± 0.00	25.5 ± 0.00	28.0 ± 1.00
5+				
6+	36.0 ± 0.00			35.0 ± 0.00
$b \pm s_b$	0.119 ± 0.009	0.293 ± 0.029	0.230 ± 0.037	0.356 ± 0.041
Age classes		*w* ± *se* (g)		
0+	12.3 ± 1.47			5.3 ± 1.45
1+	63.8 ± 2.90	53.5 ± 4.36	54.0 ± 5.02	45.7 ± 6.45
2+	119.9 ± 4.85	128.8 ± 9.73	97.0 ± 0.00	
3+	192.2 ± 8.74	209.2 ± 4.79	165.0 ± 0.00	201.2 ± 6.34
4+		277.0 ± 0.00	237.0 ± 0.00	284.5 ± 24.50
5+				
6+	640.0 ± 0.00			634.0 ± 0.00
$b \pm s_b$	2.95 ± 0.044	3.03 ± 0.094	2.66 ± 0.042	2.94 ± 0.063
Abundance (ind ha^{-1})	2917 ± 121.0	260 ± 13.0	458 ± 22.8	200 ± 10.2
Biomass (kg ha^{-1})	227.7 ± 9.45	29.1 ± 1.45	38.8 ± 1.67	28.7 ± 1.47
Mean weight (g)	78 ± 10.0	112 ± 14.5	84 ± 18.8	147 ± 39.0
Production (kg ha^{-1})	23.0 ± 0.85	5.8 ± 0.24	5.8 ± 0.02	5.1 ± 0.23
		Sl (cm)		
$b1 \pm s_b$	0.327 ± 0.015	0.293 ± 0.026	0.153 ± 0.117	0.312 ± 0.016
Breakpoint *Sl* (cm)	15.22	17.98	16.56	16.64
$b2 \pm s_b$	0.311 ± 0.009	0.367 ± 0.033	0.322 ± 0.066	0.319 ± 0.016
		w (g)		
$b1 \pm s_b$	2.793 ± 0.097	3.139 ± 0.276	1.202 ± 1.195	3.153 ± 0.165
Breakpoint *w* (g)	53.050	89.925	70.134	67.636
$b2 \pm s_b$	2.937 ± 0.079	2.527 ± 0.225	2.698 ± 0.305	3.082 ± 0.157

Using average *Sl* and *w* values for ages in brown trout samples (Table 1), it appeared that brown trout samples differed for their increased speed of growth in *Sl* (t_{Sl} = 5.730, df = 93, p < 0.05), but not in *w* ($t_w = t_{Sl}$ = 0.771, df = 25, p > 0.05) at the locality M. Whereas, the speed of growth at the locality D was not significantly different in *Sl* (t_{Sl} = 2.281, df = 93, p < 0.05), although the growth in *w* there was significantly faster in brown trout from the 2008 sample (t_w = 3.698, df = 25, p < 0.02). Attainment of sexual maturity in brown trout as revealed from breakpoints occurred at the locality M at greater *Sl* and *w* in 2008 than in 2003 (Figure 3), which means either they dominantly matured almost one year later, i.e., in the age of 2+ in 2008, than in 2003, or that only the proportion of the largest 1+ individuals attained the maturity in the same age. Attainment of maturity at the locality D in 2008 sample occurred at almost identical *Sl* and slightly smaller *w* than in 2003 sample (Table 1), meaning they matured at approximately the same age of 2+ during the six-year period. Brown trout parr grew in *Sl* at the locality M almost the same in 2008 as in 2003 (*t* = 1.133, df = 40, p > 0.05), as well as in *w* (*t* = 1.183, df = 40, p > 0.05). Whereas, after they matured, their growth in *Sl* in the 2008 sample was significantly faster (*t* = 5.737, df = 53, p < 0.01), although in *w* it was similar (*t* = 1.719, df = 53, p > 0.05) when compared to that in the mature brown trout from the 2003 sample. In the D sample, the growth of parr was similar in 2008 as in 2003, both in *Sl* (*t* = 1.346, df = 15, p > 0.05) and in *w* (*t* = 1.617, df = 15, p > 0.05), as well as that of mature brown trout both in *Sl* (*t* = 0.176, df =10, p > 0.05) and in *w* (*t* = 1.119, df = 10, p > 0.05).

Restriction analysis (RFLP) with the *SatI* endonuclease revealed that both fishes from the locality D, of the coloration that was not typical for brown trout of either the Da, or At lineage, were of the At lineage, as revealed from both 50 kb ladder and three positive controls, two of Da and one of the At lineages (Figure 5). The restriction endonuclease split the Control Region only in brown trout of the At lineage at C^{434} in two parts of the sizes 434bp and 646bp, whereas in those of the Da lineage the Control Region mtDNA remained intact (of 1080bp in length).

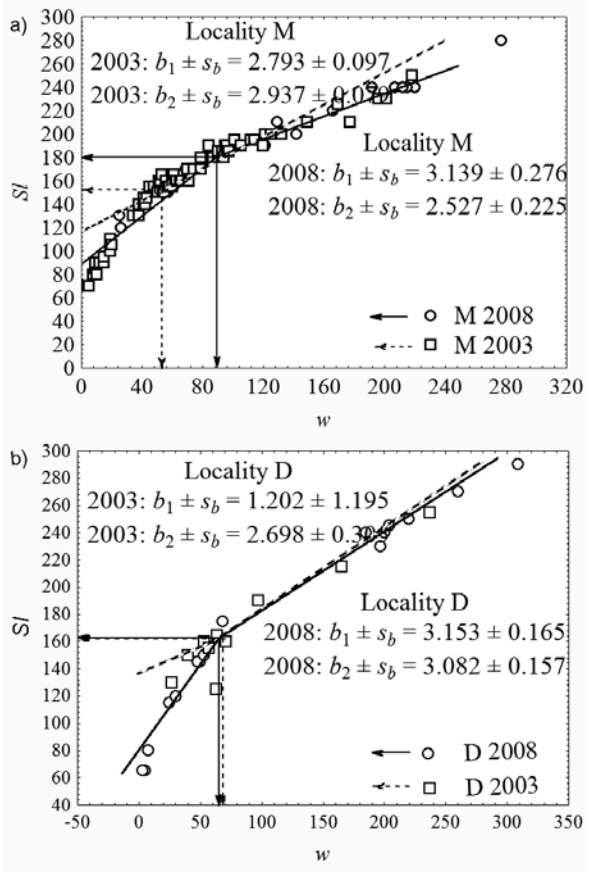

Figure 4. Breakpoints in *Sl* (cm) and in *w* (g) from the Piecewise Regression on log-transformed values at localities M (a) and D (b) in both 2008 (solid lines and hollow circle) and 2003 (dashed lines and hollow square) years of sampling, indicated on axes by arrows (for precise values, see Table 1); b_1 and b_2 are the values of linear regression slopes in brown trout at sizes before (i.e., in parr) and after maturation (i.e., in adults), respectively.

Although Welcomme (2001) stated that it is difficult to evaluate the effects of stocking in field when fish are stocked into self-reproducing population of the same species, the difference recorded in the structure of both fish community and brown trout population between years 2003 and 2008 found at one of two investigated localities of the River Gradac coincides with the difference in stocking that occurred there. In order to state unequivocally that differential stocking in the upper and lower section of the River Gradac was the main cause for that, it would be desirable to have the data about the population structure of brown trout and fish community for each year in that period, but there was no such kind of monitoring accomplished. Instead, there are some ob-

servations available from both management activities and fly fishing (e.g., CPUE and structure of brown trout in catches, personal observations). According to the records about the number of redds that were counted each year, there was an increase from less than three hundred in 2003, to almost five hundred in 2006 (pers. comm. with the fishery manager clerk); the records that give the provisional number of redds are due to difficulties in distinguishing the redds that were already counted from the new ones within the couple of days of surveillance), which indicated the strong increase in number of spawning fish during that time period. The insight into the structure of brown trout catches during the management period suggested the common structure of brown trout population in the River Gradac, with the majority of fish in lower size- and age-classes and the smaller proportion of the large, especially the trophy-size (over 50 cm *Sl*) fish. That, following the reports of fly fishermen and their wishes for the trophy-sized trout, was the driving force of the pressure on the fishery manager for the stocking with the hatchery-reared parr, but also with the brood fish in the upper course (i.e., where the locality here assigned M is situated) that occurred in the spring 2007.

At the upstream situated locality M, where the stocking with both parr and brood-size hatchery-reared brown trout occurred, the decrease in abundance of brown trout and bullhead as fellow members of that type of fish community was coupled with the increase in abundance of chub and schneider as members of the fish community that would be usually expected in the more downstream section. Appearance of chub and schneider in the upper section of the River Gradac, at the locality M is coincident with the drop in density of brown trout that are their spatial competitors and predators, respectively. That implies a likely occurrence of the empty room there. Chub inhabited pools, especially their entrance sections, whereas schneider were situated mainly in the long and calm riffles (glides), where parr of brown trout were absent from, or very rare. Constant abundance of minnow at the locality D corresponded to the much more stable structure of brown trout stock there in compare to that in the upstream section.

The drop of brown trout biomass at the locality M in 2008 was huge despite of stocking, with the strong decrease in abundance, i.e. the fading of 0+ (young-of-the-year) parr (Table 1). Management effects increased significantly the average age of brown trout at that locality, since the proportion of mature brown trout older than 2+ increased in 2008 sample at the locality D, in compare to that recorded in 2003 sample (Figure 3). The occurrence of brown trout of ages 1+ and 2+ in the M2008 sample indicates theirs survival by an attainment of the size-refuge from the predation of older and larger fish that remained in the greater proportion in that sample. The increase in average age and in abundance of larger fish in the D2008 sample implies to the shift of those categories from the upstream locality M to the downstream locality D, with the competition between the large sized brown trout as the most likely cause that acted there in addition to the predation on parr. The drop in abundance of the 0+ parr and increase in abundance of older and large-sized brown trout that resulted in an increase of average age occurring at the locality M in 2008 are in agreement with the findings of Jenkins *et al*. (1999) that large trout, whose abundance and density were suddenly augmented there by massive stocking, were competitively advantageous in compare to smaller ones when overall density increased after the brown trout parr were stocked. It would be expected that high density of mature, older and larger brown trout would slow their speed of growth, whereas the consequent drop in density of parr at locality M in 2008 (Table 1) would made possible an increase in speed of their growth in *Sl*. In contrast to expectations, there was no acceleration in growth of parr, but only in mature brown trout, and only at the locality M. That significant increase in growth of mature brown trout at the locality M is to be considered a consequence of the richness of parr, which likely were the predominant feeding resource for the stocked brood-size fish and native adult brown trout. The drop in density, i.e., in biomass and abundance and complete lack of 0+ parr in the age structure of brown trout at the locality M could be addressed to the strong predation from the heavily stocked brood-size hatchery brown trout (about 100 one-kilogram fish) in the rather short (about 4 km long) river section. The predatory pressure of that brood fish in abundance of about 25 fish km^{-1}, which is approximately one fish per each 40 m, on the parr, even without the resident large brown trout occurring there natively, should be considered heavy. This corresponds to the reports of anglers about the difference in the structure of catches of brown trout, with the abundance of

large, but slim brown trout of the weight much lower than it would be expected, as well as the scarcity of smaller brown trout in catches.

Increase in breakpoint values indicating the delay in maturation that followed the decrease of population density (relative abundance) in M 2008 samples (Figure 4) is concordant to the reports of Simonović and Nikolić (2007) that breakpoint, i.e., maturation is inversely related to brown trout density. That again justifies the use of the Piecewise Linear Regression method that derives the breakpoint between the growth periods as an approach in fisheries management as non-invasive, reliable and easy for an assessment of size at maturation. Using breakpoints could contribute to the advance in management with trout stocks, e.g., for the setting of legal landing size limit on streams and rivers where trout harvesting is to be allowed. Breakpoints are also insensitive of age-structure, because their value is dependent primarily on the alteration in the speed of growth occurring due to the investment into maturation under the state of the life-history traits in the population, and not due to only age itself. The strong increase in breakpoint values of brown trout at the locality M revealing the delay in maturation corresponded to the significant decrease in density and was accompanied with the significant increase of average age of brown trout there. At the locality D, where brown trout stock was more stable, the breakpoint values, i.e., the onset of maturation in brown trout remained almost the same.

Considering that locality M was subjected to the stocking with both parr and brood size brown trout at rather high densities of about one fish per each 4.5 m and one fish per each 40 m of river length, respectively, whereas the locality D was subjected to stocking with only par at density of about one fish per each 4.5 m, the effects recorded at the locality M are to be addressed mainly to the stocking with the large, brood size brown trout. The strong predatory effect of large, adult brook (*Salvelinus fontinalis*) and rainbow (*Oncorhynchus mykkis*) trout, especially when in high density, to the yearlings (0+) of both trout species was also reported by Larson and Moore (1985). In addition to the drop in brown trout density, the decrease in annual natural production coupled with the delay in maturation occurring at the locality M of the River Gradac could cause an extension of the brown trout stock recovery period in that river section. In contrast to that, neither the annual natural production, nor the relative biomass of brown trout of the locality D dropped. That, together with the significant increase in the speed of growth in *w*, validated that stocking with parr that was carried out according to the fisheries management plan was non-detrimental.

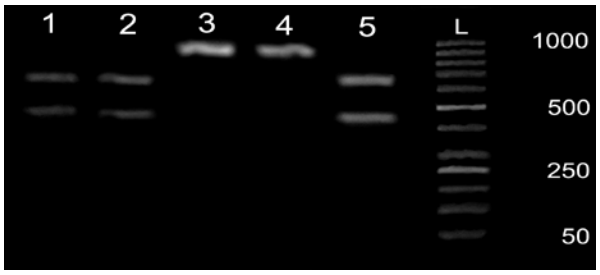

Figure 5. RFLP screening of two brown trout from the River Gradac (1 and 2).using the *SatI* endonuclease with additional three ones (3, 4 and 5) serving as controls (of Da, Da and At lineages, respectively), with the ladder of 50 bp (numbers on the right denote the size in number of bp).

Apart of the evident change in the population structure at the locality M, as well as of the finding of brown trout individual of the Atcs1 haplotype by Marić *et al* (2006), there is a direct evidence from the locality D that stockings with brown trout definitely introduced brown trout of the Atlantic lineage (Figure 4) into the gene pool of the indigenous stock of the Danubian lineage occurring in the River Gradac (Figure 4). The peculiar coloration of body (i.e., both dispersal pattern and shape of black and red spots) of brown trout landed at the locality D in 2008 sampling (Figure 4) was dissimilar both from those of Da and At lineages. The RFLP analysis using the *SatI* endonuclease revealed that both brown trout that were strange in coloration were of the At lineage, indicating that stocked brown trout of At lineage has already incorporated into the gene pool of native brown trout of Da lineage at the locality D in the lower section of the River Gradac. Considering that the River Gradac is a protected natural area, there are even more important ecological, scientific, economic, cultural and moral/spiritual reasons (Bosse, 2004) why the conservation and restoration of the native Danubian brown trout strain in it the should be undertaken. In addition to that, it is necessary to take care about both water capturing and decreased shading from riparian vegetation occurring due to timber cutting that cause the rise of summer water temperature, which could com-

promise the conservation and restoration efforts despite of the "Catch-and-Release" regime proscribed. The effective way of restoration of native stock seems the one Mitro (2004) reported an appropriate for wild trout in Wisconsin, USA. That approach was already proposed at the beginning of the period of management for the River Gradac (Simonović and Kutonova, 2004), within the frame of activities that were found appropriate for incorporation into the overall management with the declared natural protected area. It comprised the revitalization of old water mill(s) that were additionally intended to house provisional hatcheries of native brown trout, due to their infrastructural convenience for that. The lack of financial means disabled implementation of this proposal then and further management activities additionally burdened the status of the brown trout stock in the River Gradac.

Conclusion

Coincidence in change of the structure of fish community, in change of the stock of brown trout, as well as of brown trout life-history traits (age- and size-structure, abundance, density and time of maturation) that followed the stocking that involved both parr and brood fish at the upstream locality of the River Gradac, in compare to the lack of such events at the other, downstream locality where the stocking was accomplished with parr only, imply that brood fish stocked into the upper section influenced detected changes. The fishery records about the increased number of redds in spawning seasons and brown trout size distribution in catches during the five year management period that revealed the stable brown trout stock status support that. Additional effect of stocking which was recorded is introgression of the Atlantic strain of brown trout into the native stock of Danubian Lineage, as revealed from individuals of uncommon coloration.

Acknowledgement

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

STOCK ASSESSMENT OF BARTAIL FLATHEAD (*Platycephalus indicus* Linnaeus, 1758) IN NORTHWEST OF PERSIAN GULF, IRAN

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Abstract: *Platycephalus indicus* (bartail flathead) is dominant species of Platycephalidae family in Iran's southern waters, especially Khuzestan province and mainly is captured by bottom trawl and gillnet. The population biology of this species in the northwest waters of Persian Gulf (Iran) was investigated to derive information required for its management. Parameter values of the von Bertalanffy growth Function fit to size at length frequency data (males and females combined) were: k = 0.5year^{-1}, L_∞ = 62.16 cm, t_o = −0.26 years. The estimated valve of total mortality, natural mortality, fishing mortality and Exploitation ratio (males and females combined) was: Z= 2.59 year^{-1}, M= 0.77 year^{-1}, F= 1.82 year^{-1} and E= 0.70, respectively. Exploitation rate, U and Annual total stock at beginning of year were 0.64 and 1194 T respectively. Annual average standing stock, b= 420 T, MSY= 544 T and MCY=362 T were estimated respectively. Result in this study showed exploitation ratio the bartail flathead stock is lower MSY and upper MCY. The results of the study highlight critical resource base issues and provide the direction for the future management of this species in the northwest waters of Persian Gulf.

Keywords: Bartail flathead, Population biology, Assessment, Persian Gulf

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Öz:

Kuzey Batı Basra Körfezinde *Platycephalus indicus* Linnaeus, 1758′ in stok değerlendirmesi

Platycephalus indicus türü dişi ve erkek bireylerinin boy frekans verilerinden hesaplanan Von Bertalanffy büyüme eşitliği parametreleri sırasıyla k = 0,5 yıl^{-1}, L_∞ = 62,16 cm (Çatal boy), t_o=−0.26 yıl olarak bulunmuştur. Toplam Ölüm Oranı Z=2,59 yıl^{-1}, Doğal Ölüm Oranı M=0,77 yıl^{-1}, Balıkçılıktan Kaynaklanan Ölüm F=1,82 yıl^{-1}, Sömürülme Oranı E=0,70, stoka katılan birey başına düşen bağıl ürün miktarı (Y/R)' = 0,019 ve biyomasa katılan birey başına düşen bağıl ürün miktarı (B'/R) = 0,10 olarak; yıllık ortalama kalıcı stok ile ilgili parametreler ise sırasıyla, b=40 ton, MSY=544 ton ve MCY=362 ton olarak hesaplanmıştır.

Anahtar Kelimeler: *Platycephalus indicus, Populasyon biyolojisi, Stok tahmini, Basra körfezi*

Introduction

About 25 species of Platycephalidae belonging to 10 genera have been identified around the world (Smith and Heemstra, 1986). *Platycephalus indicus* (Linnaeus, 1758) is a benthic fish found on sand or muddy bottom in vary shallow area of estuary and near shore to depth of 25 m. This species is dominant species of Platycephalidae family in Iran's southern waters, especially Khuzestan province and mainly is captured by bottom trawl and gillnet (Parsamanesh *et al.*, 2000). The amount of catch for this species in Northwest of Persian Gulf was recorded as 410 and 917 tons between 2000 and 2010 (Mohammadi *et al., 2011). P. indicus* (Bartail flathead) has played economically a great role in the Northwest of Persian Gulf fishery and also known as a target species for capture in Persian Gulf region countries. It has cost about 6 US $/kg. Studies on the age and growth of *P. indicus* have been reported earlier in Persian Gulf (Nasir, 2000) in Kuwait waters ,(Marais, 1984), in Hong Kong waters (Wu, 1984) and in coastal waters of west Kyushu, Japan (Masuda *et al*., 2000). This is the first study which was carried out on the growth of *P. indicus* from Northwest of Persian Gulf waters. The present study is based on twelve months data collection from Khuzestan Coastal Waters between January 2010 and December 2010. The objectives of this study is to provide information pertaining to biological reference points and other population dynamics information required for management of this species in northwest of Persian Gulf .

Materials and Methods

The length frequency data was regularly collected from Liphe-Busafe and Bahrekan landing between 29° 44'-07'N and 48° 45' - 49° 50' W (Figure 1). A total of 469 specimens of *P. indicus* were captured between Jan 2010- Dec 2010 using bottom trawl and gill net. Total length (±1.0 mm) and weight (±0.001 g wet weight) were measured and sex recorded for each fish in the laboratory.

Parameters of the length weight relationship were obtained by fitting the power function $W = a \times TL^b$ to length and weight data where: W is the total wet weight, a is constant determined empirically, TL is the total length (Biswas, 1993). In order to verify if calculated b was significantly different from 3, the t-test was employed (Zar, 1996).

The length frequency thus collected was grouped into 10 mm class intervals. The growth estimates were made by ELEFAN employing FiSAT II program developed by Gayanilo *et al* (2002*)*. The total mortality coefficient was estimated by length converted catch curve of Pauly (1980):

$$Ln\left(\frac{N}{\Delta t}\right) = a + b \times t$$

Where, b=Z (Total mortality rate) with the sign changed. The instantaneous rate of natural mortality (*M*) was estimated using the following multiple regression model (Pauly, 1980):

Log (M) = -0.0066-0.279 log ($L\infty$) +0.6543 log (K) + 0.4634 log (T)

The mean annual environment temperature (T) used in the estimation was 23° C (according to Iran Environment Public Authority). Fishing mortality rate (F) was calculated as (Sparre & Venema, 1998): F = Z - M.

The parameter t_0 of the growth equation was estimated using the following equation (Pauly, 1980):

Log (t_0) = -0.3922- 0.2752log (L_∞) -1.038log (K)

In order to facilitate the comparison of the results with those of other studies, growth perfor-

mance index (Φ) was estimated by the following equation (Pauly and Munro, 1984):

$$\Phi = \log (K) + 2\text{Log} (L_\infty)$$

The exploitation rate (U), was estimated by: $U=F (1-e^{-z})/z$ (Pauly, 1983). The annual total stock at the beginning of the year was estimated by: Y/U where Y is the annual average catch of the species (Nurulamin *et al*., 2000). Annual average standing stock was estimated by: b= Y/F (Nurulamin *et al*., 2000). MSY was estimated by the equation: MSY=0.5×Z×B (Nurulamin *et al*., 2002 and 2004).

Figure 1. Location of two landing sites of bartail flathead in Khuzestan Coastal Waters (Iran)

MCY was estimated by the equation: MCY=2/3 × MSY (Jenning *et al*., 2000). The relative yield per recruit (Y'/R) and relative biomass per recruit (B'/R) were conducted to obtain reference points and determined the exploitation status. The model of Pauly and Soriano (1986) was used to predict the relative yield per recruit (Y/R) as follows:

$$Y'/R = EUM/k [1–(3U/1+m) + (3U^2/1+2m) + (U^3/1+3m)]$$

where: $m = (1 – E)/ (M/k) = k/Z$, $U = 1 – (L_c/ L_\infty)$, $E = F/Z$ and $B'/R = (Y'/R)/F$ (Gayanilo *et al*., 2003). The relative biomass per recruit (B'/R) was estimated by: B'/R= (Y'/R)/F (Gayanilo *et al*., 2003).

Results and Discussion

Length frequency distribution

From the total number of caught fishes, 248 were males and the remaining were females (1: 0.92). According to Table 1, mean ±S.D length values for this species were 353±180 and maximum and minimum total length was 57 mm and 1886 mm respectively. Mean ± S.D weight values were 384±130g and maximum and minimum weight were 140 g and 600 g respectively (Table 1). Average length and weight in females were higher than in males.

The length-weight relationship were calculated as $W=0.000009\ TL^{2.95}$ ($n=248$, $R^2=0.83$) for males, $W=0.000005TL^{3.07}$ ($n=198$, $R^2= 0.82$) for females and $W=0.000004TL^{3.10}$ ($n=470$, $R^2 =0.86$) for total fishes (Fig 2a,b). Verifying calculated b with 3, using t-test there was significant difference between calculated b and 3 ($P<0.05$).

Growth Studies

The growth parameters of von Bertalanffy equation (males and females combined) were as, L_∞: 62.16 cm and K: 0.5 ($year^{-1}$) and t_0: -0.26 ($year^{-1}$). The 95% confidence regions around the von Bertalanffy growth function parameter estimates for both sexes suggesting that the growth

characteristics between males and females were not similar (Table 2, Fig 3a,b).

The value of growth performance index, Φ', estimated from the growth parameters was 1.19, which gave the Von Bertalanffy growth equation for this species as: Lt=62.16 (1-exp(-0.5 (t+0.26))). The Φ' for males and females studied fishes were found to be 0.91, 1.2 respectively (Table, 2).

Table 1. Average values (±S.D.) of size corresponding of bartail flathead in Khuzestan Coastal Waters.

Month	Number of captured	Male	Female	Mean W ±S.D (g)	Min – max	Mean TL ±S.D (mm)	Min – max
January-2010	114	82	32	356±72	255-550	362±72	113-1163
February	31	23	6	294±20	224-380	179±20	64-395
March	31	11	17	280±23	201-358	153±23	57-347
April	63	40	23	361±14	266-535	389±72	114-1170
May	33	19	11	317±59	237-550	254±20	57-8410
June	30	19	11	315±43	236-550	264±23	84-1490
July	15	1	13	446±72	317-590	677±72	207-1238
August	13	2	10	357±20	280-515	418±60	141-1097
September	22	6	3	290±23	140-372	185±23	58-344
October	33	6	23	389±72	277-555	525±72	70-1540
November	35	11	23	333±88	235-490	309±40	71-817
December-2010	55	24	28	298±53	285-600	539±63	146-1886
Average	-	-	-	**130±384**	**140-600**	**180±353**	**57-1886**

Table 2. Estimate growth, mortality and yield of bartail flathead in Khuzestan Coastal Waters.

species	L_∞	K	t_0	Φ'	M	F present	Z	E present	Y'/R	B'/R
Female	64.14	0.36	0.37-	0.91	0.7	1.17	1.86	0.63	0.017	**0.1**
Male	59	0.52	-0.26	1.2	0.91	1.85	2.64	0.72	0.019	**0.1**
Both	**62.16**	**0.5**	**-0.26**	**1.19**	**0.77**	**1.82**	**2.59**	**0.70**	**0.019**	**0.1**

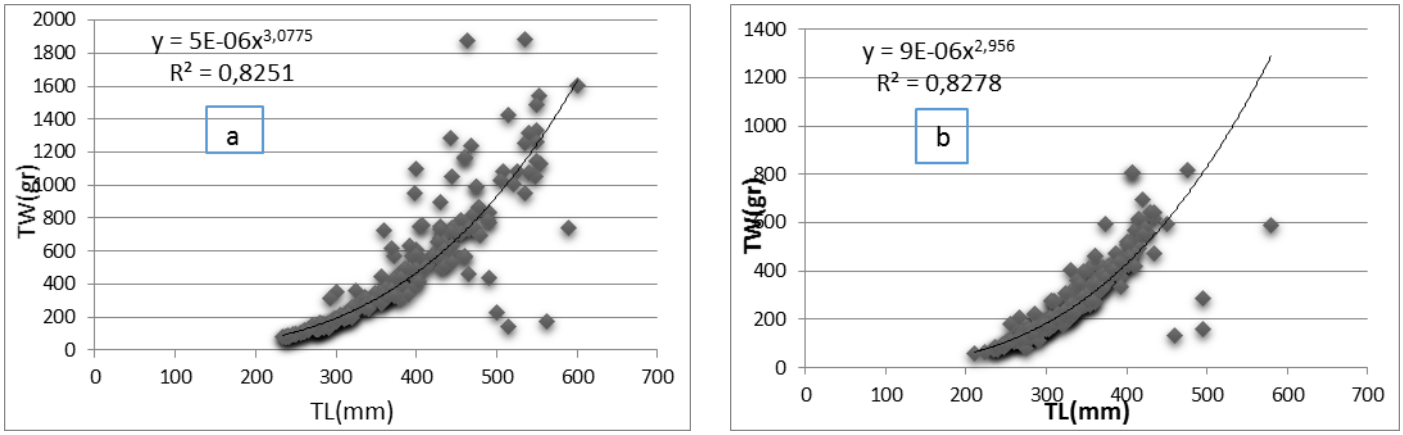

Figure 2. The length-weight relationship curve for female (a) and male (b) fish of bartail flathead in Khuzestan Coastal Waters .

Mortality estimate, relatively yield, relative biomass per recruit:

The annual instantaneous rates of fishing induced mortality (F), natural mortality (M) and total mortality (Z) are given in Table 2.

The total mortality coefficient (Z) was calculated as 2.59 year^{-1} by Pauly's length converted catch curve method (Fig 4). The annual mortality coefficient (M) was estimated as 0.77 year^{-1} by Pauly's method. The fishing mortality (F), thus obtaiend was 1.82 year^{-1}. the exploration rate was calculated as 0.70.

Fishery Assessment

The relative yield-per-recruit (Y'/R) and biomass-per-recruit (B'/R) were determined as a function of L_c/L_∞ and M/K. L_c estimated at 17.5 cm and and L_c/ L_∞ and M/K were 0.27 and 1.54 respectively. Relative yield per recruitment (Y'/R) calculated as 0.019 and relative biomass per recruitment, (B'/R) calculated as 0.10 for bartail flathead using both sex data (Fig.5). The Y'/R and B'/R for males and females is shown in Table 2. The size at which yield per recruit would be maximized (L_{max} = 29.1 cm) approximated the mean size of fish that were 0.99 years old and was considerably greater than the mean size at first capture.

Exploitation rate and annual total stock at beginning of year were calculated as 0.64 and 1194 respectively. T and Annual average standing stock, b: 420 T, MSY= 544 T and MCY=362 T were estimated respectively.

Perhaps, the earliest report on the growth study of *P. indicus* from the Persian Gulf is by Bawazeer (1989). He employed ELEFAN method and estimated the infinity length and K as 48.90 cm and 0.34 y^{-1} in Kuwait waters respectively. In the present study L_∞ and K was higher infinity length and growth coefficient which was reported by Bawazeer (1989). There are no available growth data from other studies for *P. indicus* in the studied area. The ages of flathead (*Platycephalus indicus*; Japanese name: Magochi) were (fitted to the von Bertalanffy growth equations) L_t=430.3 (1−exp(−0.667 (t+0.093))) for males, and L_t=551.5 (1−exp(−0.478 (t+0.125))) for females(Masuda *et al*., 2000). Tirasin (1993), indicated that growth parameters differed depending on species, population, age groups in the same population and even sexes. So the differences seen in different locations may be accepted as normal.

Maximum age (T_{max}) for male and female was found to be as 8.335 and 5.76 year respectively. Our results indicated that males have revealed higher growth condition and have short lifetime than female. Absorbed energy is used for body maintenance, activity, reproduction and less than 1/3 for growth. In difference species growth ratio and life cycle is different (King, 2007).

Bawazeer (1989) reported Age at zero length (t_0) of this species as calculated as -0.64 year which less than our result (-0.26). Negative t_0 values indicated juveniles grew more quickly than the predicted growth curve for adults (King, 2007). Values of Φ' for *P. indicus* has 2.91 in Kuwait waters (Bawazeer, 1989) which compere with present study show the high growth performance value.

The b values in the weight-length relationship were measured close to 3 for *p. indicus* fishes that indicating that weight increased isometric with length (King, 2007). Naik *et al* (1990) have estimated the value of b for Indian waters (the Netravati Gurpur Estuary, Mangalore) 2.99 and 2.91 for male and female respectively. Bawazeer (1989) reported the b value of weight-length was 3.32 for total fish of this species in Kuwait waters. The variation of b in the different regions could be by seasonal fluctuations in environmental parameters, physiological conditions of the fish at the time of collection, sex, gonad development and nutritive conditions in the environment of fish (Biswas, 1993).

The result of exploration ratio revealed that there is Pressure on stock of this species in northwest waters of Persian Gulf side. According to Gulland (1971, 1979), the yield is optimized when F=M. These results are important for fisheries management authorities as they suggest that the resource is heavily overexploited and in addition to a revision of mesh size regulations, a substantial reduction in fishing effort would also be required if management objectives are to be achieved (Hashemi and Kashi, 2012).

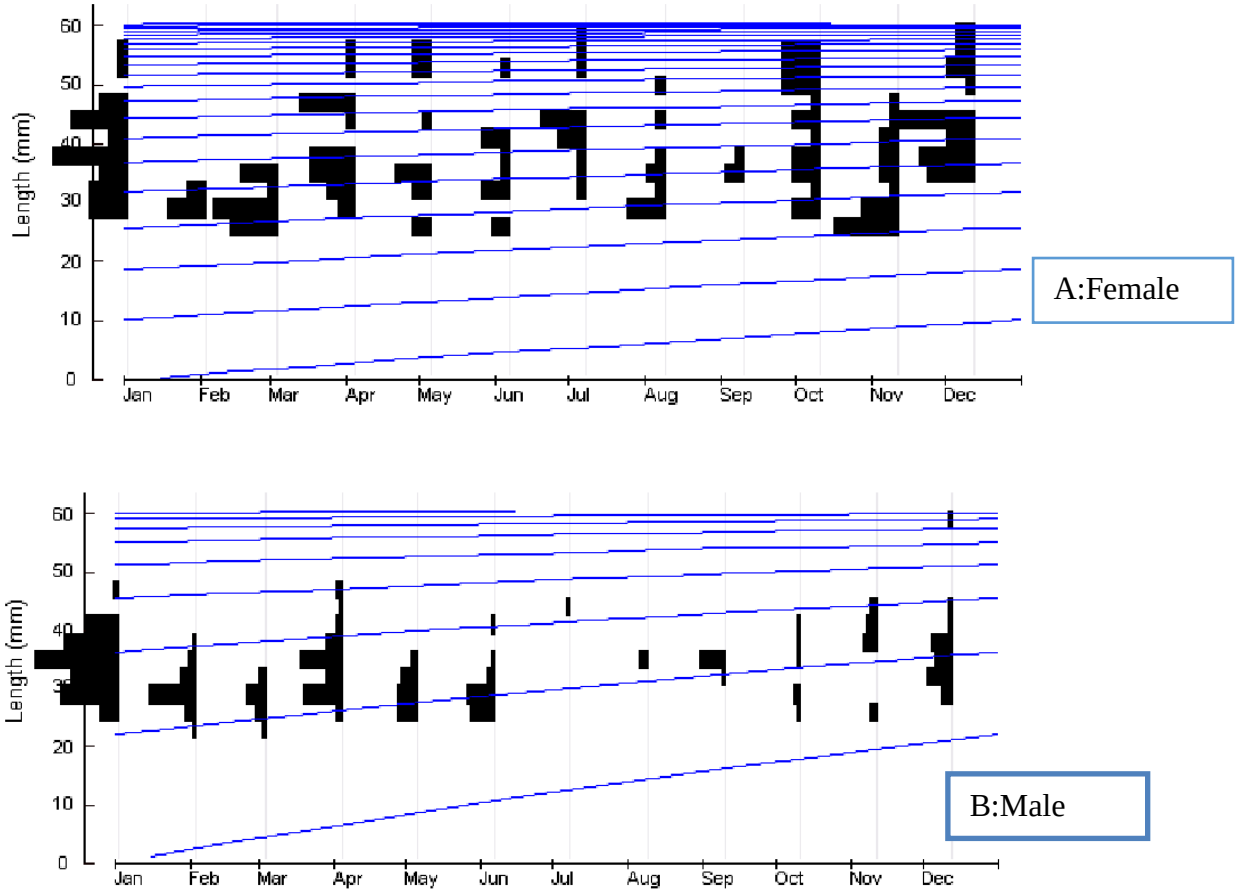

Figure 3. Growth curve of bartail flathead by ELEFAN I estimated on the restructured length-frequency diagram ($L\infty$=64.14 cm and K=0.36 yr^{-1} (A: Female) and $L\infty$=59 cm and K=0.52 yr^{-1} (B: Male)).

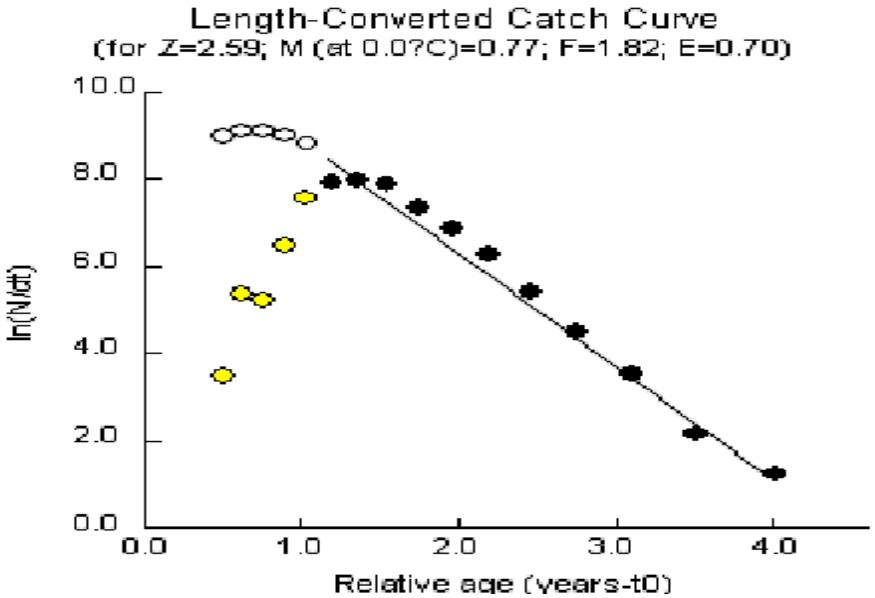

Figure 4. FiSAT graphic output of the catch curve analysis for bartail flathead.

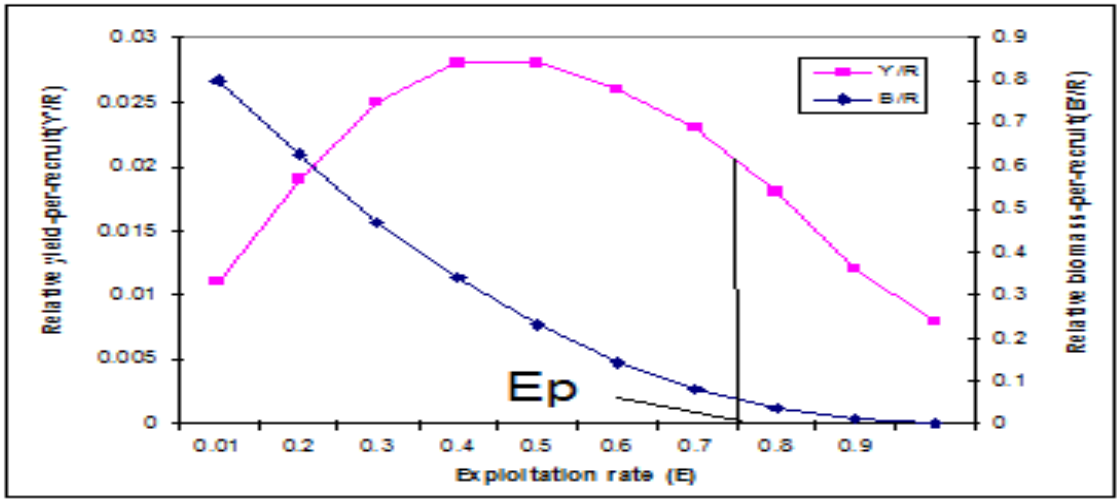

Figure 5. Relative yield and biomass per recruit curves (descending lines) for bartail flathead showing the existing exploitation rate (*E*p).

Result in this study showed exploitation ratio the bartail flathead stock is lower MSY and higher MCY. Increase in the size at first capture would be associated with an increase in yield at the existing fishing mortality rate. However, the existing fishing mortality rate (1.82 year^{-1}) was greater than that which would maximize yield per recruit, clearly demonstrate that growth over fishing is occurring for this species. The relative biomass per recruit at the estimated fishing mortality rate was particularly low at less than 15% of the unexploited level. If the critical spawning stock biomass is between 20 and 50% of the unexploited level, as suggested by King (2007), recruitment over fishing is also likely to be occurring for *P. indicus*. In conclusion, any increase in the existing fishing level/exploitation would most likely result in a reduction in the yield per recruit and thereby hamper the optimum level. It is necessary to immediately impose fishing regulation on the stock.

Acknowledgements

The present study was carried out within the framework of the research project "Determination of the ecological relationship among economic fishes in the coastal area of the Persian Gulf" funded by Iranian Fisheries Research Organization (IFRO) and Iran National Science Foundation. Special thanks for presentation of material and spiritual supportive aids and services for this national project and all colleagues that helped during field work.

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ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

DIETARY LYSINE REQUIREMENT OF JUVENILE EURASIAN PERCH (*Perca fluviatalis*)

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Abstract: The aim of this study was to estimate the optimum dietary lysine requirement of juvenile Eurasian perch (*Perca fluviatalis*), and to investigate the influence of different dietary lysine levels on growth, carcass composition, somatic indices and haematocrit value. A total of 240 juvenile perch was included in a 12-week feeding study. Based on the whole body amino acid profile of in total 48 perch of three different weight groups, eight semi-purified diets containing graded levels of lysine (L1-L8) were produced. The diets contained approximately 12% crude lipid and 40% crude protein and the lysine level varied from 12.2 (L1) to 24.3 (L8) g kg^{-1} dry matter (DM). Approximately 40% of the total nitrogen content in the diets was in the form of protein-bound amino acids. The ideal protein concept was applied for estimation of the amino acid requirements. Fish fed diets L5-L8 (18.3-24.3 g lysine kg^{-1} DM) had higher final body weight, weight gain, protein gain and specific growth rate ($P<0.001$) than fish fed diets L1-L4 (12.2-17.3 g lysine kg^{-1} DM). Dietary lysine level did not significantly affect intraperitoneal fat, haematocrit value and carcass composition. The results indicate that the lowest lysine level required for optimal growth performance in Eurasian perch is 18.3 g lysine kg^{-1} DM.

Keywords: Eurasian perch, Lysine requirement, Essential amino acids, Growth, body composition, Ideal protein

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Öz: **Jüvenil Tatlısu Levreği (*Perca fluviatalis*)'nin Diyet Lizin İhtiyacı**

Bu çalışmanın amacı jüvenil tatlısu levreği (*Perca fluviatalis*)'nin optimum diyet lizin ihtiyacının belirlenmesi ve diyetinde bulunan farklı lizin düzeylerinin büyüme, karkas kompozisyonu, somatik indeksi ve hematokrit değeri üzerine etkilerinin incelenmesidir. Bu çalışmada toplamda 240 jüvenil tatlısu levreği 12 hafta boyunca süren yemleme çalışmasına dâhil edilmiştir. Üç farklı ağırlık grubundan 48 tatlısu levreği örneğinin tüm vücut aminoasit profiline göre sekiz adet farklı düzeylerde lizin içeren yarı-saf diyet (L1-L8) grubu oluşturulmuştur. Diyetler, yaklaşık olarak %12 ham lipit, %40 ham protein ve kuru maddede (KM) 12.2 g/kg (L1) ile 24.3 g/kg arasında değişen miktarlarda lizin içermektedir. Diyetlerdeki toplam azotun yaklaşık %40'ı proteinlere bağlı aminoasitler formundadır. Aminoasit ihtiyacının belirlenebilmesi için ideal protein konsepti uygulanmıştır. L5-L8 arasındaki diyet gruplarıyla (18.3 – 24.3 g lizin /kg KM) beslenen balıklarda, L1-L4 (12.2 – 17.3 g lizin /kg KM) arasındaki diyet gruplarıyla beslenen balıklara göre deney sonunda daha yüksek vücut ağırlığı, ağırlık artışı, protein kazanımı ve özgül büyüme oranı ($P<0.001$) elde edilmiştir. Diyet lizin seviyesinin, karın içi yağ miktarı, hematokrit değer ve karkas kompozisyonunu belirgin biçimde etkilemediği görülmüştür. Bu çalışmanın sonuçları, tatlısu levreğinde optimum büyüme performansı için ihtiyaç duyulan en düşük lizin seviyesinin 18.3 g lizin /kg KM olduğunu göstermiştir.

Anahtar Kelimeler: Tatlısu levreği, Lizin ihtiyacı, Esansiyel amino asitler, Büyüme, Vücut kompozisyonu, İdeal protein

Introduction

Eurasian perch (*Perca fluviatalis*) has been identified as a potential species for fish culture (Kestemont and Dabrowski, 1996). There is a high market demand for perch in Europe and the catch of wild perch is not sufficient to meet this demand. One of the identified bottlenecks to a profitable culture of perch is the lack of information about its nutrient requirements. Few nutritional studies have been conducted with Eurasian perch this far, and diets are not standardised. Fiogbe et al. (1996) studied the effects of crude protein (CP) level in juvenile Eurasian perch, and concluded that 370-440 g CP kg^{-1} diet results in the most effective nutrient utilisation. Previous studies have examined the optimal dietary protein to energy ratio in yellow perch (*P. flavescens*) (Ramseyer and Garling, 1998) and the protein requirement is reported to be below 300 g CP kg^{-1} diet (Brown et al., 1996; Ramseyer and Garling, 1998; Schaeffer et al., 2011). This is markedly lower than the requirement reported for Eurasian perch.

Several studies have investigated the dietary amino acid (AA) requirements of yellow perch with regard to the AA composition of the ovaries, muscle and whole body (Ramseyer and Garling, 1994), dietary arginine and sulphur AA requirements (Twibell and Brown, 1997; 2000), optimal dietary AA profile (Hart et al., 2010) and the effect of a lysine-supplemented diet (Kwasek et al., 2012). However, to our knowledge there are no published data on the AA requirements of Eurasian perch.

Lysine is the AA found in highest concentrations in the carcass of several fish species (Wilson and Cowey, 1985; NRC, 2011) and it is the first-limiting indispensable AA (IAA) in most protein sources used for commercial feed production (Hauler and Carter, 2001). When combined with the ideal protein concept, the lysine requirement can be used to predict the requirements of other IAA in an animal species. The ideal protein concept has been applied for estimation of the AA requirements of fish, based on the whole-body tissue of a given fish species (Green and Hardy, 2002; Rollin et al., 2003; Furuya et al., 2004). The ideal protein concept proposes that the optimum dietary IAA requirements be considered as proportions relative to total IAA, usually expressed as per cent of lysine.

The objectives of this study were to assess the dietary lysine requirement of juvenile Eurasian perch fed a semi-purified diet using growth performance, somatic indices and blood haematocrit value as response parameters; and to estimate the requirement for the other IAA (except tryptophan) using the ideal protein concept. The working hypothesis was that the dietary AA requirements of Eurasian perch are different from those of yellow perch.

Materials and Methods

Fish and facilities

The first generation offspring of Eurasian perch collected as eggs in the wild were obtained from a commercial fish hatchery (Östgös AB, Söderköping, Sweden) and brought to the Swedish University of Agricultural Sciences, Uppsala, Sweden. The fish were stored in a 1000-L flow-through tank and fed a commercial diet (nutrient composition, g kg^{-1} DM: crude protein: 410-450; crude lipid: 130-170; ash: 60) to apparent satiation twice daily. The health status of the fish was checked by the National Veterinary Institute, and no anatomical, pathological or histological anomalies were found. One week prior to experimental start 240 fish (41.7 ± 8.1 g; mean ± SD) were randomly placed in groups of 10 in 24 experimental units (consisting of 200-L glass aquariums divided in two by a sponge filter) organised into three floors. Fish within each floor were randomly assigned to one of eight treatments (L1-L8). The experimental aquariums were connected to an indoor recirculating system with mechanical and biological filtration, and UV treatment. Water replacement rate in the system was about 20% h^{-1}. Each aquarium was supplied with two water inlets, recirculating water and freshwater (water flow 5.0 L min^{-1} and 1.0 L min^{-1} respectively; 22-23 °C), water outlets at bottom and top and continuous aeration. Feed waste and faeces were removed every second day from the tanks, which were totally cleaned once a week manually with a vacuum cleaner. During the experiment water conditions were monitored weekly. Dissolved oxygen concentration ranged from 7.4 to 8.5 mg L^{-1} (HQ 40d, Hach Lange GmbH, Berlin, Germany), pH from 7.5 to 8.0, ammonium-nitrogen (N) from 0.0 to 0.5 mg L^{-1} and nitrate-N from 25.0 to 50.0 mg L^{-1} (below the detection limit for nitrate-N) (Sera aqua-test box, Heinsberg, Germany). The diurnal light: dark cycle was 12:12 h. The experiment was performed in compliance with laws and regulations on procedures and experiments in live animals in Sweden, which are overseen by the Swedish Board of Agriculture.

Diets and feeding

Prior to the experiment the amino acid profile of the whole body, excluding viscera, of 48 farmed Eurasian perch fed a commercial diet (Table 1) was analysed. Fish were classified into three groups according to body weight: small (mean 7.6 g, n=40), medium (69.8 g, n=4) and large (420.2 g, n=4). Based on these findings, eight semi-purified, iso-nitrogenous and iso-energetic experimental diets containing graded levels of lysine (diet code L1-L8) were produced. Fish meal, casein and gelatine were used as protein sources, and were supplemented with mixtures of dispensable amino acids (DAA) and indispensable amino acids (IAA), excluding lysine (Table 2). Approximately 40% of the total N content in the diets was in the form of protein-bound amino acids. To maintain iso-nitrogenous diets, L-glutamic acid was used to balance diets L1-L7 on a molal (mol kg^{-1}) basis. Minerals and vitamins were added to the diets in accordance with earlier studies on feeding semi-purified diets to yellow perch (Twibell and Brown, 1997). The diets were produced by cold-pelleting in a meat grinder (MR9-TC22, Nima maskinteknik AB, Örebro, Sweden) (3.5 mm die) and the strains formed were dried (forced air oven; 50 °C, 24 h), chopped and screened (2.5 mm) to obtain an appropriate size of pellets. The pellets were stored at -25 °C until feeding.

During the adaption period, the fish were fed a commercial diet by hand to apparent satiation twice daily. During the first week of the experimental period, fish were fed the experimental diets by hand to apparent visual satiation, to allow them to adapt to the diets and to estimate the apparent visual satiation. Excess rations (100% of satiation=full ration) of each diet were constantly fed for 11 hours a day, 6 days a week, with automatic feeders. The feed allowance of each tank was adjusted every third week.

Due to a high stress response in the fish at cleaning, we were unable to collect feed wastes to a satisfactory level. Consequently, feed intake had to be excluded from our data, which made it impossible to calculate the actual quantities of protein and lysine ingested.

Sampling

Before the start of the experiment with graded levels of lysine, fish fed the commercial diet and classified into three groups according to body weight were sacrificed and stored at -25 °C for later analysis of initial whole body (except viscera) (Table 1). Prior to the feeding of the experimental diets, all fish were mildly anaesthetised (tricaine methanesulfonate, MS222; Western Chemical Inc., Ferdale, WA, USA) and individually weighed. At the end of the experiment, all fish were anaesthetised (MS222), individually weighed, a blood sample was collected from the caudal vein by cutting off the caudal fin and the fish were finally killed by cutting the brachial arches. Blood was collected in Na-heparinized micro haematocrit tubes and centrifuged at 14 000×g for 2 minutes for determination of haematocrit value (Hct). The fish were then dissected and liver, intraperitoneal fat and final parts of viscera removed and weighed for calculation of the hepatosomatic index, viscerosomatic index and intraperitoneal fat ratio. For whole body composition analysis, three fish from each tank were randomly selected.

Chemical analysis

Feeds and fresh carcasses were homogenised (B-400, Büchi Labortechnik AG, Flawil, Switzerland), freeze-dried and ground through a 1-mm screen (Tecator Cyclotec 1093, Höganäs, Sweden) and then stored at -25 °C. Feeds and carcasses were analysed for dry matter (DM) (103 °C for 16 h in a well-ventilated oven, cooling in desiccator before weighing) and ash (550 °C for at least 3 h and until the ash had a white colour, cooling in desiccator before weighing), and total nitrogen (N) was determined by the Kjeldahl method using a 2020 digestor and a 2400 Kjeltec Analyser unit (FOSS Analytical A/S, Hilleröd, Denmark). The crude protein content was calculated as N×6.25 (Nordic Committee on Feed Analysis, 1976). Amino acid content of feeds and fish was analysed by high-performance liquid chromatography at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden), as described by Llames and Fontaine (1994). Briefly, samples were oxidised with performic acid for 16 h prior to hydrolysis for 23 h with 6N HCl. Individual amino acids were separated on an ion-exchange chromatograph (Biochrom 30 amino acid analyser, Biochrom Ltd., Cambridge, England) and the peaks were identified, integrated and quantified with EZChrom Elite (Biochrom Ltd., Cambridge, England). Crude lipid was determined according to the Official Journal of the European Communities (1984), using a 1047 Hydrolysing Unit and a Soxtec System HT 1043 Extraction Unit (FOSS Analytical A/S, Hilleröd, Denmark). Gross energy was determined with an isoperobol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA; Table 3).

Calculations

Weight gain (WG) and specific growth rate (SGR) were used as indicators for growth and protein gain (PG) was used as an indicator for protein utilisation. WG, SGR and PG were calculated according to the following equations:

$$WG\ (\%)=(FW-IW)\times 100/IW$$

$$SGR\ (\%\ day^{-1})=(\log FW-\log IW)\times 100/day$$

$$PG\ (\%)=(FW\times CP_F/100-IW\times CP_I/100)\times 100/(IW\times CP_I/100)$$

where FW is the final body weight (g), IW is the initial weight (g), CP_F is the final crude protein content in the body (%) and CP_I is the initial crude protein content in the body (%). The relative weights of liver, intraperitoneal fat and viscera were expressed as hepatosomatic index (HSI), viscerosomatic index (VSI) and intraperitoneal fat (IPF), and were calculated according to the following equations:

$$HSI\ (\%) = (W_{Liv}/LW)\times 100$$

$$VSI\ (\%) = (W_{Vis}/LW)\times 100$$

$$IPF\ (\%) = (W_{IntrF}/LW)\times 100$$

where LW is the live weight of the fish (g), W_{Liv} is the weight of the liver (g), W_{Vis} is the weight of the viscera (g) and W_{IntrF} is the weight of the intraperitoneal fat (g).

Statistical analysis

The statistical analysis was conducted using the SAS programme version 9.3 (SAS Institute, Inc, Cary, NC, USA). The effect of fish size on carcass composition of perch fed a commercial diet was evaluated with Proc GLM, with fish size (small, medium and large) as fixed factors. The effect of experimental diet on growth, survival, carcass composition, body indices and haematocrit value was evaluated with Proc Mixed, followed by the Tukey's Multiple Comparison test. The model included the fixed factor of treatment (lysine level) and the random factor of aquarium. The effect of floor was also tested as a fixed fac-

tor, but no significant effect was found and it was therefore excluded from the model. When treatments L1-L4 were compared with treatments L5-L8 the model included the fixed factor of group. Aquarium was used as the experimental unit for growth performance, body composition and survival, whereas fish was used as the experimental unit for HSI, VSI, IPF and Hct. The level of significance was set at $P<0.05$ and a tendency at $P<0.10$.

Results and Discussion

Whole body analyses of the perch fed the commercial diet showed that large perch had a lower protein content in their body than small and medium-sized perch ($P<0.001$) (Table 1). The fat and ash content was also lower in large perch than in small and medium-sized perch ($P<0.001$ and $P=0.012$, respectively). The lysine content was highest in small fish ($P=0.005$), as was the content of histidine, leucine and phenylalanine.

The fish ate well during the whole experiment. A total of 25 fish died (10.4%), but there was no relationship between dietary treatment and mortality. Growth performance, somatic indices, survival rate and haematocrit change of juvenile perch fed the experimental diets are summarised in Table 4. The mean final weight was 64.6±16.0 g, with minimum and maximum weights of 35.9 and 114.6 g, respectively. Fish fed diets L6 and L8 had a higher FW ($P<0.001$) than fish fed diets L1, L2, L3 and L4. There was a tendency for fish fed diet L4 to have a lower FW than fish fed diet L5 ($P=0.069$). Fish fed the highest inclusion level of lysine had higher WG ($P=0.049$; Table 4) than fish fed the lowest inclusion level. The WG of fish in treatment L4 was unexpected low. The PG was poorer in fish fed diets L1-L4 than in those fed diet L8 ($P=0.002$; Table 4). SGR did not differ between treatments with the exception of fish fed diets L3 and L8 ($P=0.005$). However, there was a tendency for fish fed diet L8 to have a higher SGR than fish fed diets L1 and L4 ($P=0.057$ and $P=0.067$, respectively), and for fish fed diet L3 to have a lower SGR than those fed diets L5 and L6 ($P=0.075$ and $P=0.085$, respectively).

Comparison between treatments L1-L4 and treatments L5-L8 resulted in significant differences in FW (51.6 vs. 69.7 g), WG (44.9 vs. 65.4%), PG (27.5 vs. 47.9%) and SGR (0.44 vs. 0.60% day^{-1}) ($P<0.001$ for all).

Fish fed diets L5, L6 and L8 had a lower HSI than fish fed diets L1 and L4 ($P=0.001$). Fish fed diets L5-L8 had a lower VSI than fish fed diet L1 ($P=0.043$). There was no effect of dietary treatment on IPF and Hct. When treatments L1-L4 were compared with treatments L5-L8 significant differences in HSI (2.8 vs. 2.3) and VSI (11.1 vs. 10.4%) ($P<0.001$ and $P=0.004$ respectively) were observed.

Feeding juvenile perch the experimental diets did not affect ($P>0.05$) the whole body composition. Across treatments, the body composition was: DM (g kg^{-1}) 282.2±19.8 ($P=0.622$, s.e.=1.21); crude protein (g kg^{-1} DM): 675.5±12.0 ($P=0.683$, s.e.=0.79); crude lipid (g kg^{-1} DM): 125.7±7.8 ($P=0.836$, s.e.=0.46) and ash (g kg^{-1} DM): 216.4±8.0 ($P=0.255$, s.e.=0.52).

The average IAA composition in the whole body of fish from all groups fed the commercial diets were calculated and are presented together with published values for yellow perch, rainbow trout (*Oncorhynchur mykiss*) and Arctic charr (*Alpinus salvelinus*). Based on the values from the present study, the IAA requirements of Eurasian perch were estimated using the ideal protein concept (Table 5).

In our study fish fed 18.3 g lysine kg^{-1} DM or more had a higher weight gain (WG) and protein gain (PG) than fish fed diets containing less lysine. The response in WG and PG to increasing lysine content in the diet was not proportional at the lowest levels of inclusion. In addition, there were large variations in growth response between replicates which may be related to poor feed intake in individual fish within replicates. Undomesticated perch is sensitive towards stress, which may reduce growth due to the increased energy consumption and a lower feed intake (Jentoft et al., 2005; Strand et al., 2007). Thus, due to large variation between replicates and few replicates we were unable to get an acceptable fit of our data to the broken line model or the saturation kinetic model for WG and PG as a function of lysine level. However, the lowest lysine level (18.3 g kg^{-1} DM) resulting in improved protein gain in our study was slightly lower compared with values reported for other species; 20.0 and 22.0 g kg^{-1} DM in Atlantic salmon (*Salmo salar*, Anderson et al., 1993; Espe et al., 2007); 21.0 g kg^{-1} DM in Asian sea bass (*Lates calcarife*r, Murillo-Gurrea et al., 2001); 20.0 g kg^{-1} DM in milkfish (*Chanos chanos*, Borlongan and Benitez, 1990) and 23.2 g kg^{-1} DM in silver perch

(*Bidyanus bidyanus*, Yang et al., 2011). However, the value found in the present study is higher than the dietary lysine requirement reported for hybrid striped bass (*Morone chrysops* X *M. saxatilis*); 14.0 g kg^{-1} DM (Griffin et al., 1992) and Nile tilapia (*Oreochromis niloticus*); 14.3 g kg^{-1} diet (Santiago and Lovell, 1988). It is possible that our estimate of the dietary lysine requirements could be slightly overestimated as the utilization of crystalline amino acids can be lower than the utilization of protein-bound amino acids. However, on the contrary, if the fish did not reach their maximum growth capacity, the obtained requirement might be slightly underestimated. Therefore, the result from this study needs to be verified under practical conditions.

Several authors have reported that protein utilisation is a more sensitive parameter of dietary lysine deficiency than weight gain (Gahl et al., 1991; Rodehutscord et al., 1997; Encarnacao et al., 2004; Grisdale-Helland et al., 2011). In addition to protein, body weight gain includes lipids, ash and water, which will affect the precision of the estimates if the retention of these compounds differs between treatments. Gahl et al. (1995) demonstrated that for pigs, the responses in protein and lysine gain approached a plateau at a considerably higher level (120 or 145% of the estimate lysine requirement) than the response in weight gain, which was observed at approximately 100% of estimated lysine requirement. A higher response in protein and lysine gain compared with weight gain has also been reported in Atlantic salmon (Grisdale-Helland et al., 2011), but not in rainbow trout (Kim et al., 1992b), black sea bream (*Sparus macrocephalus;* Zhou et al., 2010*)* or grass carp (*Ctenopharyngodon idella*; Wang et al., 2005). However, in the present study, the correlation between dietary lysine content and protein gain (r = 0.50) was similar to that of weight gain (r = 0.48).

Table 1 Carcass composition of three different sizes of Eurasian perch (g kg^{-1} DM)

	Fish size				
	Small[1]	Medium[2]	Large[2]	s.e.	P-value
Proximate analysis					
Crude protein	721[a]	717[a]	657[b]	5.33	< 0.001
Crude fat	102[a]	106[a]	151[b]	3.45	< 0.001
Ash	178[a]	169[a]	192[b]	7.31	0.012
Indispensable amino acids					
Arginine	42.9[a]	42.0[ab]	41.4[b]	0.30	0.026
Histidine	18.3[a]	17.3[b]	16.9[b]	0.24	0.008
Isoleucine	29.6	28.4	26.8	0.68	0.052
Lysine	56.9[a]	53.8[b]	52.4[b]	0.74	0.005
Methionine	20.4	19.6	18.4	0.19	< 0.001
Phenylalanine	26.7[a]	25.4[b]	24.7[b]	0.27	0.002
Leucine	49.0[a]	47.1[b]	44.8[c]	0.49	< 0.001
Threonine	29.4[a]	28.5[ab]	27.5[b]	0.33	0.008
Valine	32.3	31.4	29.9	0.59	0.061
Sum	305.5	293.5	282.8		
Dispensable amino acids					
Alanine	46.5	46.7	46.3	0.39	0.716
Aspartic acid	67.6[a]	65.1[b]	63.5[b]	0.53	0.001
Cysteine[3,4]	7.3	6.9	6.4	0.11	0.004
Glutamic acid	100.7[a]	96.5[b]	92.1[c]	0.92	< 0.001
Glycine	55.9[a]	61.4[b]	61.2[b]	1.01	0.006
Proline	33.1	34.9	35.8	0.88	0.159
Serine	31.1	30.3	29.3	0.52	0.100
Tyrosine[4]	20.7[a]	18.7[b]	18.5[b]	0.28	< 0.001
Sum	362.9	360.5	353.1		

Values within the same row with different superscripts are significantly different (P<0.05).
Data are presented as least square means. s.e. = pooled standard error.
[1]Mean values, n = 40.
[2]Mean values, n = 4.
[3]Cysteine = amount present after oxidation of cysteine and cystine to cysteic acid.
[4]Conditionally indispensable (NRC, 2011).

The SGR in the present study varied between 0.41 and 0.63% day^{-1}, which is an acceptable growth rate. Few studies have been published regarding SGR of Eurasian perch of the same size as used here (mean initial weight = 41.7 g). A SGR of 0.5% day^{-1} for 15.8-22.7 g perch housed under similar conditions as in the present study was reported by Melard et al. (1996); Kestemont et al. (2001) and Fiogbe and Kestemont (2003). Furthermore, Cho et al. (1992) and Kim et al. (1992a; b) obtained acceptable growth with diets containing high levels of crystalline amino acids. However, Peres and Oliva-Teles (2005) showed that if high levels of dietary protein (more than 19%) were replaced with crystalline amino acids in juvenile turbot (*Scophthalmus maximus*) diets the growth performance was depressed. It has been shown in several fish species that crystalline amino acids are more rapidly absorbed, and/or absorbed earlier in the alimentary canal than protein-bound amino acids (Yamada et al., 1981b; Kaushik and Dabrowski, 1983; Cowey and Walton, 1988; Zarate and Lovell, 1997; Zarate et al., 1999). This might lead to a slight metabolic dyssynchrony with amino acids derived from protein digestion and a greater proportion of the crystalline amino acids being catabolised, probably resulting in a temporarily higher concentration of amino acids in tissues or plasma (Cowey and Walton, 1988; Zarate et al., 1999). To avoid this metabolic dyssynchrony in our study, the feed was provided at very short time intervals. Yamada et al. (1981a) showed that more frequent feeding intervals increase weight gain and metabolic utilisation of crystalline amino acids in common carp (*Cyrpinus carpio*). In contrast, Zarate et al. (1999) did not notice any interactions between protein-bound lysine and crystalline lysine fed at different feeding frequencies to channel catfish (*Ictalarus punctatus*).

Table 2 Ingredient composition of experimental diets (g kg^{-1} DM)

	Diet code							
	L1	L2	L3	L4	L5	L6	L7	L8
Fish meal	85.0	85.0	85.0	85.0	85.0	85.0	85.0	85.0
Fish oil	114.0	114.0	114.0	114.0	114.0	114.0	114.0	114.0
Casein	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
Gelatine	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Dextrin[1]	250.0	250.0	250.0	250.0	250.0	250.0	250.0	250.0
Carboxymethyl cellulose	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
α-cellulose	73.3	72.7	72.3	71.8	71.5	71.0	70.5	70.0
IAA mix[2]	199.3	199.3	199.3	199.3	199.3	199.3	199.3	199.3
DAA mix[3]	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
L-Lysine-HCl[4]	0.0	2.6	5.1	7.7	9.0	11.6	14.1	16.7
L-Glutamic acid[4]	13.5	11.4	9.3	7.2	6.2	4.1	2.1	0.0
Titanium dioxide[5]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mineral premix[6]	80.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Vitamin premix[7]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

[1]Maltodextrin (dextrose equivalent 7.5).

[2]Indispensable amino acid mix (% of mixture): L-arginine-HCl 7.28, DL-methionine 8.03, L-threonine 12.49, L-methionine 8.03, L-Tryptophan 8.33, L-histidine 8.03, L-isoleucine 12.29, L-leucine 20.17, L-phenylalanine 10.79, L-valine 12.59, and Evonik Degussa International AG.

[3]Dispensable amino acid mix (% of mixture): DL-alanine 29, L-aspartic acid 9, L-glutamic acid 31,L-glycine, EVONIK Industries AG.

[4]EVONIK Industries AG.

[5]Titanium dioxide (TiO_2) was added as an inert marker for digestibility. Due to problems in sampling, digestibility was not calculated.

[6]Mineral premix consisted of (g kg^{-1} premix): SeO_2, 0.3; $CaSO_4$, 350; NaH_2PO_4 *H_2O, 200; KH_2PO_4, 200; $MgSO_4$ * $7H_2O$, 20; $MnSO_4$ * $4H_2O$, 2; $FeSO_4$ * $7H_2O$, 2; $CuSO_4$ * $5H_2O$, 1.5; NaCl, 12; $ZnSO_4$ * $7H_2O$, 2; $Ca(IO_3)_2$ *H_2O, 0.1; $CoCl_2$ * $6H_2O$, 0.1; Na_2MoO_4 * H_2O, 0.5; $AlK(SO4)_2$ * $12H_2O$, 2; NaF, 0.7.

[7] Vitamin premix consisted of (I.U. or g kg^{-1} premix): Retinyl palmitate, 800 000 I.U.; Cholecalcipherol, 100 000 I.U.; RRRα-tocopherol acetate, 20; Menadione, 1; Thiamine, 1.5; Riboflavin, 2; Pyridoxine,1; Cyano cobalamine, 0.0002; Ascorbic acid, 20; Phantothnic acid, 5; Niacinamide, 15; Folic acid, 0.7; Biotin, 0.025; Choline chloride, 370; Inositol, 10.

The experimental diets in the present study were formulated according to diets previously used to yellow perch by Twibell and Brown (1997), in which casein and gelatine were used as sources of intact protein. The diets were formulated to give a high feed intake and a good growth rate, and to contain the maximum level of fish meal to maximise palatability without exceeding the lowest target level of the total lysine content in the experimental diets. Brown et al. (1993; 1996) reported low feed intake for purified diets containing no or low levels of fish meal and fish oil in experiments with hybrid striped bass and yellow perch, respectively. In addition, Peres and Oliva-Teles (2005) reported that the voluntary feed intake was lower for juvenile turbot fed diets with high level of crystalline amino acids. However, the feed in the present study was not rejected by the fish, but the feed intake was lower than in earlier experiments with Eurasian perch fed commercial or fish meal-based diets (Langeland, unpublished data). This lower intake was probably due to some unpalatable feed ingredients. Eurasian perch seems to be sensitive to the palatability of the feed and therefore this should be taken into consideration when formulating semi-purified diets for perch. However, Twibell and Brown (1997) stated that a purified diet remains an appropriate choice for determining nutritional requirements.

The body composition of perch fed the experimental diets did not differ between groups. This is in line with results from studies in other fish species fed graded levels of lysine (Borlongan and Benitez, 1990; Peres and Oliva-Teles, 2008), indicating that dietary lysine does not affect body composition. However, Kim et al. (1992b), Encarnacao et al. (2004) and Marcouli et al. (2006) found a positive correlation between lysine intake and body protein content in fish fed lysine-deficient diets. The ideal amino acid profile of Eurasian perch was compared with that of yellow perch and with the mean value for 10 teleost fish species (Figure 1). The IAA profile of Eurasian perch is very similar to that of yellow perch, but differs from that of rainbow trout and Arctic charr. However, in the present study the viscera was excluded from the whole body analysis, whereas in the reported studies the viscera was included. Thus, this may affect the comparison with other species.

A lower HSI in fish fed diets with high levels of lysine compared to fish fed lysine-deficient diets has been reported by several authors for different fish species; European sea bass (*Dicentrarchus labrax*, Tibaldi et al., 1994), gilthead sea bream (*Sparus aurata*, Marcouli et al., 2006), turbot (Peres and Oliva-Teles, 2008) and silver perch (Yang et al., 2011). In the present study HSI was lower in fish fed the highest level of lysine than in fish fed the lowest level of lysine. Between the other treatments no clear pattern could be observed. The higher levels of HSI found in the fish fed low lysine level diets might be due to lysine limitation in protein synthesis. The AA which are not used to deposit protein are deaminated and metabolised to lipids or glycogen and stored in the liver. In this experiment the Hct value was unaffected by different levels of lysine. In contrast, Yang et al. (2011) showed that the Hct value in silver perch was lower in fish fed lysine-deficient diets.

Table 3 Analysed diet composition (g kg^{-1} DM or MJ kg^{-1} DM)

	Diet code							
	L1	L2	L3	L4	L5	L6	L7	L8
Dietary component								
Crude protein	404.4	398.3	401.9	401.5	403.8	405.6	405.6	406.0
Crude lipid	115.8	119.8	116.3	118.9	117.3	119.3	117.5	119.0
Ash	69.3	71.7	68.2	69.7	70.3	71.5	73.8	71.2
Gross energy	19.9	19.7	19.9	19.8	19.7	19.7	19.9	19.8
Indispensable amino acids								
Arginine	21.7	21.8	21.8	21.6	22.2	21.8	21.6	21.9
Histidine	18.3	18.5	18.4	18.5	18.5	18.3	18.4	18.3
Isoleucine	31.6	31.7	32.2	31.7	32.0	32.1	32.0	31.9
Leucine	50.9	50.5	51.1	50.7	51.0	50.9	50.6	50.9
Lysine	12.2	13.5	15.3	17.3	18.3	20.1	22.1	24.3
Methionine	18.3	18.1	18.0	18.1	17.8	18.0	17.9	17.8
Phenylalanine	26.8	26.8	27.3	26.9	27.1	26.9	27.2	27.2
Threonine	28.7	29.1	29.2	29.0	29.8	28.8	28.9	29.1
Valine	31.8	32.0	23.7	32.2	32.7	32.7	32.6	32.8
Sum	240.3	242	237	246	249.4	249.6	251.3	254.2
Dispensable amino acids								
Alanine	21.9	22.6	23.3	22.3	22.6	22.4	22.2	22.1
Aspartic acid	17.0	16.7	16.9	16.8	16.8	16.8	16.9	16.8
Cysteine[1,2]	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Glutamic acid	59.0	55.4	53.9	50.9	51.0	48.7	45.9	44.6
Glycine	27.3	27.9	28.0	28.6	28.5	28.5	28.1	27.4
Proline	16.2	15.7	15.8	14.8	16.9	15.0	15.8	16.0
Serine	8.3	7.9	8.0	7.8	8.4	8.2	8.0	8.1
Tyrosine[2]	6.2	5.8	5.8	5.7	5.9	5.5	5.7	5.9
Sum	156.7	152.8	152.5	147.7	150.9	145.9	143.4	141.7

[1]Cysteine = amount present after oxidation of cysteine and cystine to cysteic acid.
[2]Conditionally indispensable (NRC, 2011).

Table 4 Growth, survival, somatic indices and haematocrit value of Eurasian perch fed diets containing graded levels of lysine

	Diet code									
	L1	L2	L3	L4	L5	L6	L7	L8	s.e.	P-value
Initial weight (g)	41.4	41.1	41.4	40.9	41.8	44.2	39.8	43.0	1.32	0.421
Final weight (g)	59.8^{ab}	60.9^{ab}	58.5^{a}	59.1^{ab}	69.0^{bc}	72.8^{c}	64.4^{abc}	72.8^{c}	2.12	<0.001
WG (%)	44.2^{a}	48.6^{ab}	41.7^{a}	44.9^{ab}	65.3^{ab}	64.6^{ab}	62.0^{ab}	69.7^{b}	5.19	0.005
PG (%)	28.0^{ab}	30.6^{ab}	24.3^{a}	27.2^{ab}	47.2^{bc}	47.7^{bc}	43.7^{abc}	53.1^{c}	4.63	0.002
SGR (% day^{-1})	0.44^{ab}	0.47^{ab}	0.41^{a}	0.44^{ab}	0.60^{ab}	0.59^{ab}	0.57^{ab}	0.63 ^{b}	0.04	0.005
Survival (%)	90.0	93.3	86.7	90.0	90.0	93.3	93.3	86.7	5.65	0.968
HSI (%)	3.0^{a}	2.7^{ab}	2.5^{ac}	3.0^{a}	2.2^{bc}	2.1^{c}	2.6^{ac}	2.1^{c}	0.20	0.001
VSI (%)	11.7^{a}	11.0^{ab}	10.7^{bc}	11.2^{ab}	10.6^{bc}	9.9^{c}	10.5^{bc}	10.5^{bc}	0.36	0.043
IPF (%)	3.7	4.0	3.3	3.5	3.8	3.7	3.9	4.3	0.21	0.068
Hct (%)	36.7	37.0	42.5	35.1	37.9	40.0	36.9	37.3	1.75	0.121

Values within the same row having different superscripts are significantly different ($P < 0.05$).
Data are presented as least square means. s.e. = pooled standard error.
Abbreviations: WG, weight gain; PG, protein gain; SGR, specific growth rate; HSI, hepatosomatic index; VSI viscerosomatic index; IPF, intraperitoneal fat and Hct, haematocrit value.

Table 5 Indispensable amino acid content (g kg^{-1} DM) in whole body of Eurasian perch, yellow perch, rainbow trout and Arctic charr, and estimated IAA requirements of Eurasian perch using the ideal protein concept

Amino acid	Eurasian perch[1]	Yellow perch[2]	Rainbow Trout[3]	Arctic charr[4]	IAA requirements of Eurasian perch[1]
Arginine	42	41	64	63	14.2
Cysteine[5]	7	4	-	-	2.3
Histidine	18	18	29	25	5.9
Isoleucine	28	29	43	31	9.5
Leucine	47	47	76	70	15.8
Lysine	54	53	85	89	18.3
Methionine	20	18	29	28	6.6
Phenylalanine	26	29	44	48	8.6
Threonine	29	27	48	50	9.6
Tryptophan	-	-	-	-	-
Tyrosine[5]	19	20	9	-	6.5
Valine	31	33	51	41	10.5

[1]Data from present study.
[2]Data from Ramseyer and Garling (1994).
[3]Data from Arai (1981).
[4]Data from Gurure et al. (2007).
[5]Conditionally indispensable (NRC, 2011).

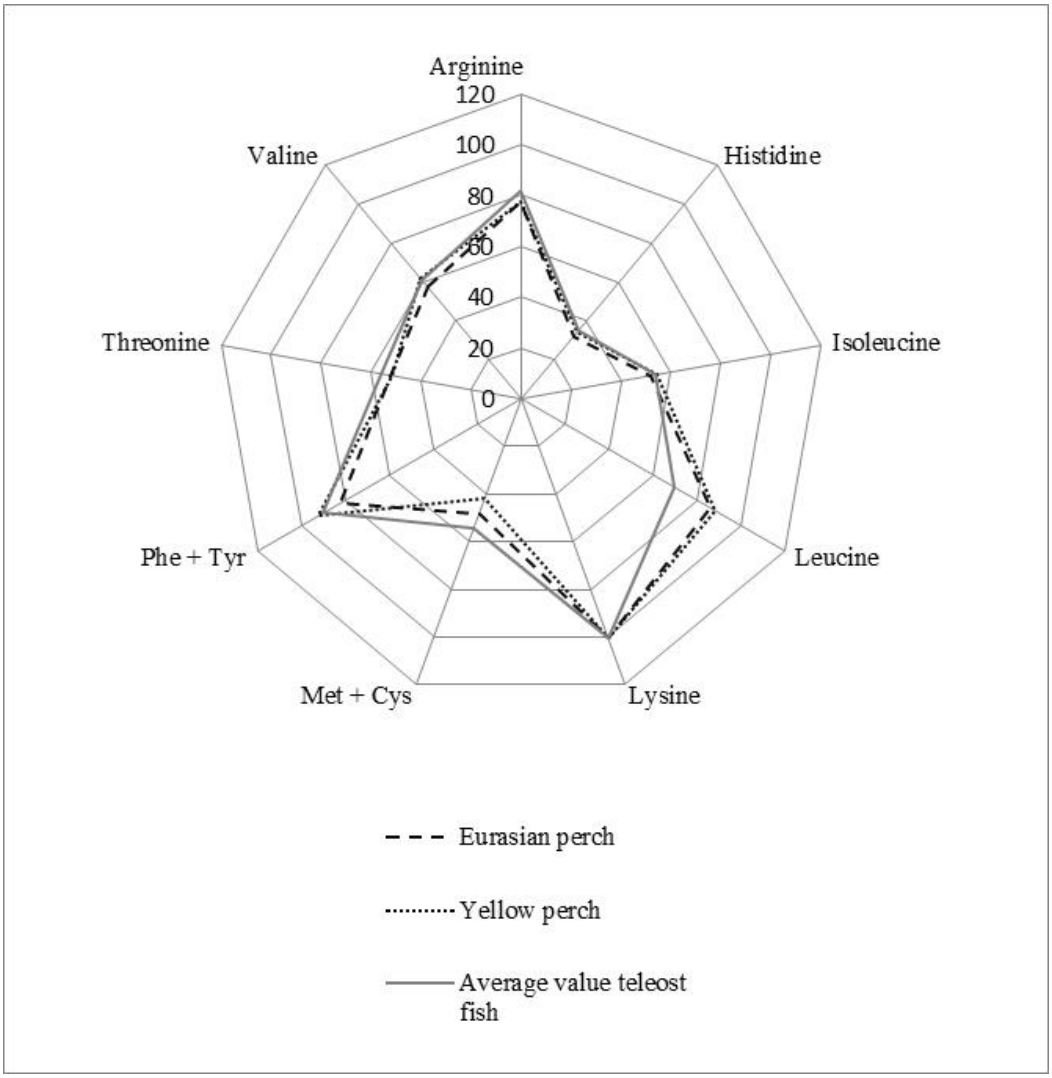

Figure 1. Ideal amino acid profile for Eurasian perch (this study), yellow perch (Ramseyer and Garling, 1994) and average value for 10 teleost fish species (NRC, 2011).

Conclusions

The estimated minimum dietary lysine requirement for optimal weight gain and protein gain of juvenile Eurasian perch appears reasonable compared with other published data on Atlantic salmon, Asian sea bass, milkfish and silver perch. However, before the predicted dietary IAA requirements of Eurasian perch presented in this paper could be used as a general recommendation for feed formulation the result should be verified in further studies.

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