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The effect of transportation stress on tissue ascorbic acid levels of Mexican silverside (*Chirostoma estor estor* Jordan, 1979)

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The Atherinopsid silverside (*Chirostoma estor estor* Jordan, 1879) is a native freshwater species from the Central Mexican Plate. It is the principal species in an artisanal fishery in Lake Pátzcuaro, one of the altiplano lakes of Central Mexico, where for many years it has been the basis of the subsistence of the riparian P'urépecha Indian people. It has a high market demand and a high commercial value and so is an important income source in the region. However, problems like pollution, introduction of exotic species and overfishing have caused it to become an endangered species.

C. estor estor has a great potential for aquaculture, and although there have been several attempts at culture on experimental scales (Lara, 1974; Armijo and Sasso, 1976), research on the basic requirements for its culture began only recently. Martínez-Palacios *et al.* (2002a) described techniques for measuring and handling *C. estor estor* larvae and determined the optimum temperature for growth and survival. Other studies have been conducted on physiology, feeding habits, anatomical feeding structures and activity of digestive enzymes of the species (Martínez-Palacios *et al*., 2002b) and the effects of saline environments on growth and survival of eggs and larvae of whitefish are now well known (Martínez-Palacios *et al.,* 2004). Currently, several studies are in progress on feeding requirements, *in vivo* and *in vitro* digestibility of different food sources, molecular biology and stress responses in order to establish the optimal conditions for aquaculture.

The species is very easily stressed by handling and changes in environmental conditions such as salinity and dissolved oxygen, result in high mortality, low growth, lack of appetite, etc. According to Pankhurst and van der Kraak (1997), stress can inhibit growth because of its effect on metabolism and the endocrine pathways that regulate this process.

It has been shown that vitamin C (ascorbic acid) plays an important role in resistance to environmental stress in fish (Wee, 1996; Papp *et al.*, 1997; Li *et al.,* 1998; Henrique *et al.*, 1998; Montero *et al.*, 1999), as well as acting on numerous physiological processes including growth, bone formation, reproduction, wound healing, and immune responses, amongst others (Lygren

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et al., 1999; Lim *et al.,* 2000; Lee and Dabrowski, 2004). One of the most widely accepted functions of vitamin C is that it acts as an antioxidant as it interrupts free radical chain reactions.

Vitamin C occurs at high levels in many vital organs with an active metabolism and its concentration in various tissues is related to the dietary intake of this vitamin. In some tissues, such as the brain, it is accumulated in high concentrations and, compared with other storage organs such as liver and kidney, its levels can be retained for longer. It seems that Vitamin C has a great importance in protecting vital tissues against oxidation processes, and this may explain the high levels found in brain and other organs such as thymus in fish (Gabaudan and Verlhac, 2001). According to Robinson (1989), the best tissue for evaluating ascorbic acid status in fish is debatable. Blood ascorbate levels are rapidly affected by dietary vitamin C intake, thus these levels not appear to be a good indicator of the vitamin C status. Anterior kidney ascorbate levels have been suggested as a good indicator of ascorbic acid reserves in fish, but this tissue is small, which causes problems for vitamin C assays. Some authors consider liver ascorbate levels to be a better indicator of ascorbic acid status than anterior kidney vitamin C levels. Lim and Lovell (1978) found highly variable ascorbate concentrations in anterior kidney of catfish, while concentrations of this vitamin in liver were consistent. In other species like Rainbow trout (*Onchorhynchus mykiss*) the liver ascorbic acid concentration can be used as an index of the vitamin C status (Hilton *et al.,* 1977).

We evaluated the ascorbic acid concentration in brain, liver, gonad and muscle of several wild *Chirostoma estor estor*[1] . Total ascorbic acid (TAA), reduced ascorbic acid (AA) and Dehydro-ascorbic acid (DHAA) concentrations were evaluated by HPLC techniques[2] (Fig. 1). Tissue ascorbate (TAA, AA and DHAA) levels were different with brain having the highest values, followed by gonad, liver and muscle in both sexes. No significant difference ($p>0.05$) exists in TAA concentrations of the evaluated tissues between females and males (Table 1), nor are there significant differences between females and males in AA and DHAA concentrations, except in brain, which has higher levels of AA in males. As shown in Figure 2, both in females and males, vitamin C in the evaluated tissues is found in higher proportion as AA (reduced form) than DHAA (oxidized form), with the exception of female brain although this difference was not significant ($p>0.05$).

During changes in environmental conditions ascorbic acid fluctuations can occur in fish tissues which suggests that vitamin C is involved in certain physiological adaptation mechanisms. In some species like mullet (*Mugil cephalus* L.), during periods of stress, tissue ascorbate concentrations can be significantly altered (decrease or increase) (Thomas, 1984). However, there is no data on vitamin C status in *C.estor estor* and the effects of stress on AA concentrations in tissues.

TABLE 1.

Total Ascorbic Acid tissue levels (μg/g) found in wild *Chirostoma estor estor* females and males. Data are expressed as mean ± S.E.M.

Tissue	Females	Males
Brain	121.81 ± 21.68^a	110.69 ± 15.92^a
Gonad	38.58 ± 11.58^a	81.43 ± 24.61^a
Liver	25.86 ± 2.94^a	46.21 ± 14.68^a
Muscle	11.23 ± 3.68^a	12.88 ± 2.80^a

Values with the same superscript are not significantly different ($p>0.05$).

[1] *Chirostoma estor estor,* (Standard length = 14.4 ± 2.03 cm) were collected in lake Pátzcuaro, Michoacán, México in September 2003. Six males and six females were sacrificed, in order to extract the brain, liver and gonad and take samples of muscle, which were frozen in liquid nitrogen within 1 minute. Frozen tissues were then stored at -80ºC until ascorbic acid analysis.

[2] Frozen tissues were weighed and immediately homogenized in ice-cold buffer (0.2 M Sodium acetate solution, pH 4.8). Samples of each homogenate (100 μL) were added to three tubes, one for the base line (BL), one for reduced ascorbic acid (AA) and the last for total ascorbic acid (TAA) quantification. The scheme of sample preparation is shown in Figure 1. Quantification of ascorbic acid concentration in the prepared samples was carried out by high performance liquid chromatography (HPLC), using the method modified by Papp *et al.* (1998). Then, dehydro-ascorbic acid (DHAA) concentrations were determined by the difference between TAA and AA. Ultrapure chemicals used in the analysis were obtained from J.T. Baker (USA), Fluka (Switzerland), SIGMA (Germany) and Aldrich (USA), and Durapore (Millipore) membrane filters (0.45 μm) were used for the filtration of buffers and homogenates. A Liquid chromatograph (isocratic single column system, Agillent 1100 series) was used with UV-visible detector and a Lichrosorb RP-18 column (4.6 x 200mm, Agillent). The mobile phase was 0.04 M Sodium acetate solution, with EDTA (0.05 mM) and Tetrabutylamonium dihydrogen phosphate (0.5 mM), pH 3.76. The chromatographic conditions were: Flow rate= 0.5 ml/min; Pressure= 65 bar; Wave length (UV detector)= 254 nm; Temperature= 23ºC; Injection volume= 2 μL. The chromatograms were evaluated with ChemStation Plus software (Agillent).

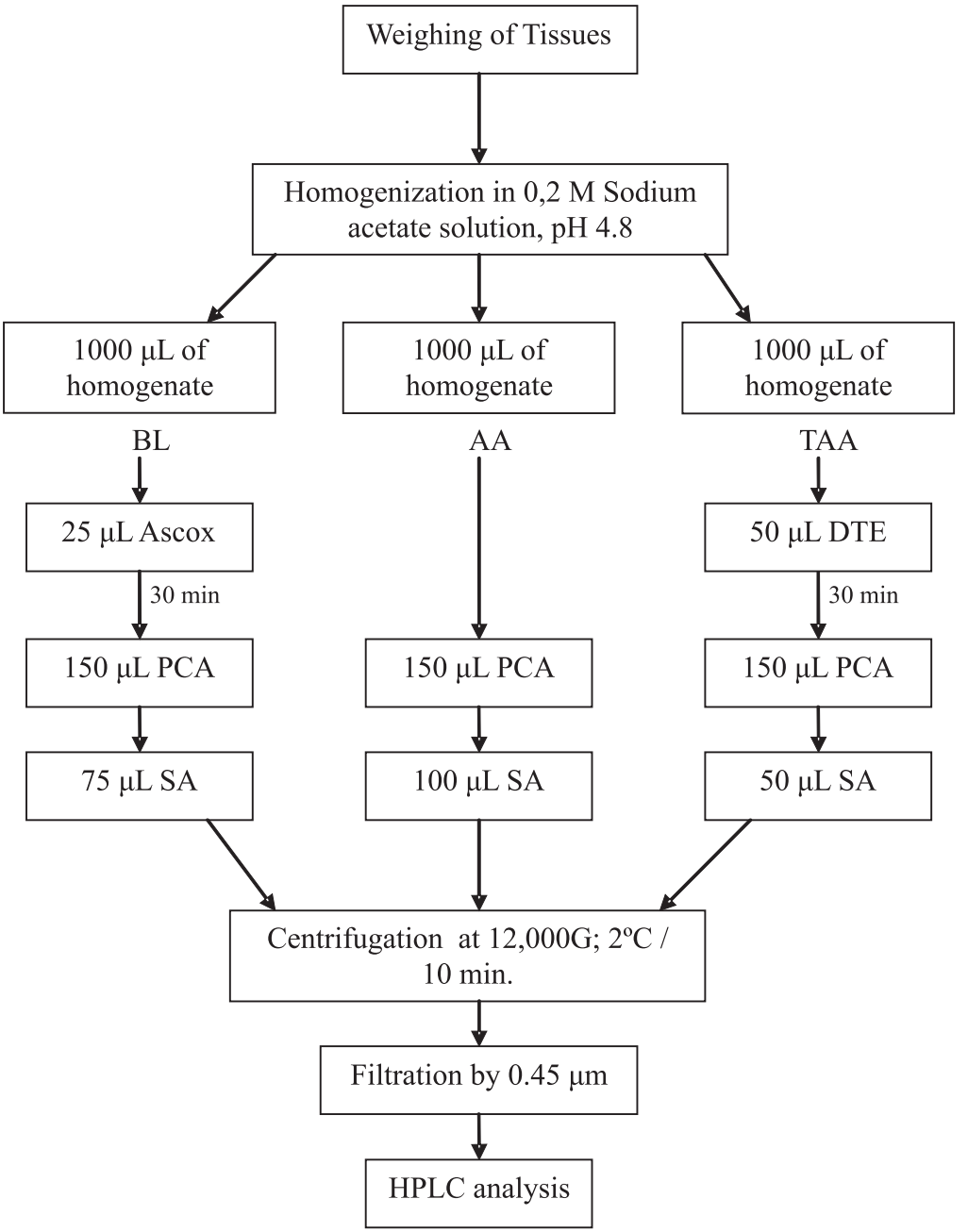

FIGURE 1. Sample preparation for the determination of ascorbic acid by HPLC method. (BL: Base line determination, AA: reduced ascorbic acid quantification, TAA: Total ascorbic acid quantification, Ascox: Ascorbate oxidase, DTE: 4% Dithioerythritol solution, PCA: 10% Perchloric acid solution, SA: 0.2 M Sodium acetate Buffer).

We monitored variations in ascorbic acid concentrations after transport stress in brain, liver, gonad and muscle of several *C. estor estor* adults. We took these fish as the stressed group and the fish sampled previously, without transport stress, as the control group[3]. After transport stress, both females and males show the same pattern of tissue ascorbic acid (TAA, AA and DHAA) as the controls, with the highest values in brain and the lowest in muscle. In comparison with pre-stress vitamin C levels there are not significant changes ($p>0.05$) in tissue ascorbate concentrations (Fig. 3).

No significant difference was found between females and males in tissue TAA concentrations after stress, with the exception of liver which had lower levels in females than males. As in the control group, post-stress vitamin C was higher in both sexes as AA than DHAA. However, there was no significant difference between these two forms in the brain of females. There is a trend of an increase of AA levels and a decrease of DHAA levels in all tissues of both sexes after the transport stress (Fig. 4), although the change in their proportion is only significant ($p<0.05$) in female gonads. We can conclude that the stress caused by three hours transportation is not enough to cause significant changes in tissue TAA concentrations, but it is sufficient to change significantly the proportion of AA and DHAA in gonad of females.

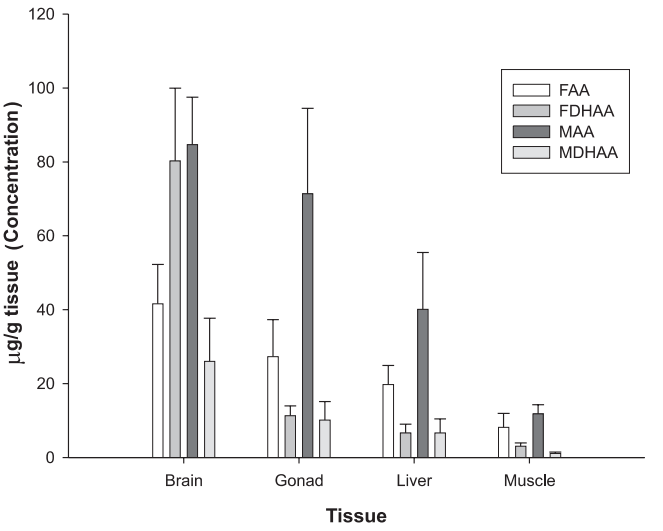

FIGURE 2. Reduced ascorbic acid (AA) and Dehydro-ascorbic acid (DHAA) levels in both females and males of wild *Chirostoma estor estor* (FAA: Females reduced ascorbic acid; FDHAA: Females Dehydro-ascorbic acid; MAA: Males reduced ascorbic acid; MDHAA: Males Dehydro-ascorbic acid).

Context and perspectives

Because the evaluated fishes were taken from the wild, it was inevitable that physical stress was caused during capture; however, it is not known if such stress caused significant changes in the normal tissue ascor-

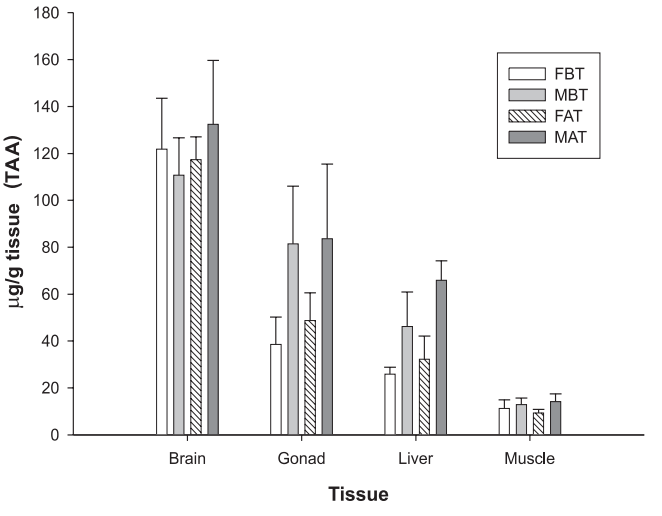

FIGURE 3. Total Ascorbic Acid (TAA) levels found in the evaluated tissues of *Chirostoma estor estor* females and males before (control group) and after transport stress (stressed group) (FBT: Females before transport; MBT: Males before transport, FAT: Females after transport, MAT: Males after transport).

[3] A further six males and six females (Standard length = 17.7 ± 1.7 cm) were packed alive in plastic bags with oxygenated freshwater and then transported for 3 hours at 22-24ºC to the laboratory. This group of fish was captured at the same time as the control group and so capture stress was the same for the two groups. After transport, fish were sacrificed and samples of brain, liver, gonad and muscle were taken and frozen in liquid nitrogen within 1 minute. Frozen tissues were then stored at -80ºC until ascorbic acid analysis.

For each tissue, the mean and standard error (S.E.) were calculated separately for males and females, before and after transport stress. For each tissue, differences in AA, TAA and DHAA before and after transport and differences between sexes were analysed using Student's t-test and the Minitab Statistical software, version 13.32. The accepted level of significance was 0.05. When data did not have a normal distribution, statistical analysis were performed using the Mann-Whitney test with a confidence level of 95%. Because the fish were captured from the wild, differences in sizes was unavaiodable; in this case the obtained data were reported as micrograms of ascorbate per gram of tissue (µg/g).

bate levels. Thomas (1984) found that liver and brain ascorbate contents were relatively unaffected by capture stress. It was assumed that capture stress does not alter the vitamin C stores in these tissues in *C. estor estor* and that fish sampled without transport stress were a valid control group.

As has been found in several fish species, highest concentrations of TAA were found in brain tissue in *C. estor estor* and these levels were maintained after the transport stress, confirming that in brain tissue the protection against oxidative damage is very important with vitamin C acting as an electron receptor (Gabaudan and Verlhac, 2001). According to Patro and Patnaik (1979) in *Ophiocephalus punctatus* the ascorbic acid concentrations in brain is unaffected by short-term exposure to physical stress, which agrees with the results of this study. Thomas (1984) also found this pattern in the brain and liver of mullet *Mugil cephalus* L. after short-term capture stress. In *C. estor estor* transport stress of three hours did not alter the ascorbate content of this tissue.

Hilton *et al.* (1977) have proposed liver ascorbic acid content as a reliable indicator of vitamin C status in fish, and considered liver ascorbate concentrations of 20 µg/g or below to be an indicator of poor vitamin C status in rainbow trout. Lim and Lovell (1978) considered levels of liver ascorbate below 30 µg/g as indicative of vitamin C deficiency in catfish. Robinson (1989) found that levels of ascorbic acid below 30µg/g did not reflect a vitamin C deficiency in channel catfish and that levels of 16.5 and 18.2 µg/g did not show signs of vitamin C deficiency. As shown in Table 1, adult *C. estor estor* had liver ascorbate levels of 25.9 µg/g (females) and 46.2 µg/g (males), which most probably indicates a good vitamin C status. Because the concentration of vitamin C in tissues is related to the dietary intake of the vitamin, the liver ascorbate concentrations found may reflect its natural feeding based on zooplankton, a natural supply of this vitamin. Nonetheless, further data are needed to establish hepatic ascorbic acid levels, which could be used as indicators of vitamin C deficiency in this species. Liver TAA levels in silverside were not affected by transport, which suggests that this physical stress may not significantly influence the vitamin C status of this fish.

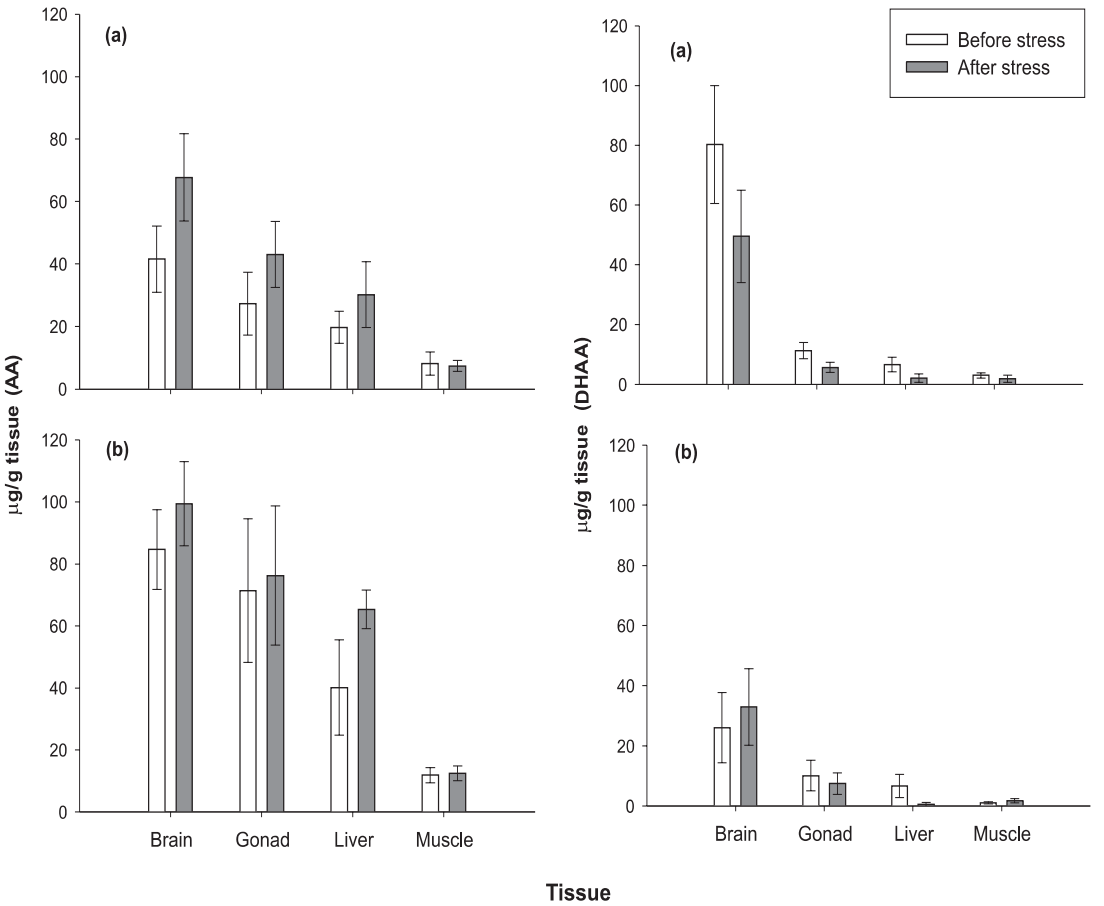

FIGURE 4. Reduced Ascorbic Acid (AA) and Dehydro-ascorbic Acid (DHAA) tissue levels found in *Chirostoma estor estor* (a) females and (b) males before (control group) and after transport stress (stressed group).

As has been reported in other scurvy-prone fish species (Table 2), *C. estor estor* has significant amounts of ascorbic acid in the gonads, to guarantee the high quality of gametes. For the control group, there were no differences of TAA concentrations in gonads between males and females. Most of the ascorbic acid found in testis was in the reduced form, while in ovaries there were no significant differences between reduced and oxidized forms. Although TAA level was not influenced by sex in *C. estor estor*, the proportion of reduced and oxidized forms did vary in gonads. In North Sea dab (*Limanda limanda)* Saborowski *et al*. (1997) found differences in AA concentrations between male and female gonads and, as was found in other species (*Carassius carassius*, *Gadus morhua, Perca flavescens*), these levels varied with the reproductive cycle (Seymour, 1981; Sandnes and Braekkan, 1981; Dabrowski and Ciereszko, 1996). In *C. estor estor*, although the evaluated gonads were immature (gonadosomatic indices between 0.11 and 0.25% for males, and between 0.27 to 0.47% for females), these tissues had higher values than liver and muscle. Different concentrations of vitamin C may be expected in mature individuals, depending on the stage of the reproductive cycle. Transportation stress did not affect the TAA levels in either males or females gonads, but the proportion of AA and DHAA changed significantly in ovaries with AA (reduced form) increasing and DHAA (oxidized form) decreasing. This suggests that vitamin C would have acted as an electron receptor, effectively protecting against oxidative damage in ovaries in a stress situation.

The lowest concentrations of TAA were found in muscle of *C. estor estor*, indicating that this tissue is not an important storage place for vitamin C. Low muscle TAA levels have been observed in other fish such as european sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata* L.) and rainbow trout (Alexis *et al.,* 1999; Gabaudan and Verlhac, 2001). The stress caused by transport for three hours did not affect muscle ascorbate levels of silverside.

Although the changes in tissue TAA levels after the stress are not significant, low variations did exist and it may be that longer periods of transport stress could result in more significant changes.

TABLE 2.

Ascorbic acid (AA) concentrations in gonads of different wild scurvy-prone fishes

Sex Species	Size	GSI (% body weight)	AA concentration ($\mu g\ g^{-1}$)	Reference
Females				
Catla catla	4.66±1.44 Kg	NA	286.34±16.54	Agrawal and Mahajan (1980)
Labeo rohita	2.45±0.83 Kg	NA	225.30±15.36	
Cirrhina mrigala	2.75±0.54 Kg	NA	206.81±8.94	
Gadus morua	NA	0.5-20	100-450	Sandnes & Braekkan(1981)
Salvelinus alpinus	12-14 cm	7.5-20	200-344	Dabrowski (1991)
Chirostoma estor estor	13.77±2.46 cm	0.27-0.47	38.58 ± 11.58	This work
Males				
Catla catla	4.66±1.44 Kg	NA	209.56±20.21	Agrawal and Mahajan (1980)
Labeo rohita	2.45±0.83 Kg	NA	199±12.64	
Cirrhina mrigala	2.75±0.54 Kg	NA	135.75±10.24	
Salvelinus alpinus	12-14 cm	8-10	60-90	Dabrowski (1991)
Chirostoma estor estor	15.54±1.01 cm	0.11-0.25	81.43 ± 24.61	This work

GSI: Gonadosomatic index
NA: Not analized

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